Allelopathic effects of submerged macrophytes on phytoplankton: determining the factors of phytoplankton sensitivity and detection of new modes of action

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Zusammenfassung

Durch Eutrophierung verloren viele der mitteleuropäischen Flachseen den von Makrophyten dominierten Klarwasser-Zustand und gingen in den Phytoplankton dominierten trüben Zustand über. Allelopathische Substanzen, die von aquatischen Makrophyten exudiert werden, können das Wachstum des Phytoplanktons inhibieren und damit potenziell zu einer Stabilisierung des Klarwasser-Zustandes in eutrophen Gewässern führen. Die vorliegende Arbeit trägt zur Aufklärung der ökologischen Relevanz dieser allelopathischen Effekte der Makrophyten auf das Phytoplankton bei, indem die Wirkmechanismen allelopathischer Substanzen (Kapitel II) und Faktoren, die die Sensitivität des Phytoplanktons beeinflussen (Kapitel III-V), erörtert werden.

Die Signifikanz allelopathischer Effekte von Makrophyten gegenüber dem Phytoplankton auf Ökosystemebene ist umstritten. Einen Hauptgrund hierfür stellen die bisher etablierten Nachweismethoden dar, welche Einschränkungen für die Messung allelopathischer Effekte *in situ* aufweisen. Daher gelang es bisher nicht, Allelopathie aquatischer Makrophyten unter *in situ* Bedingungen zweifelsfrei von anderen Effekten zu separieren. Problematisch ist insbesondere der Ausschluss einer Nährstoffkonkurrenz zwischen Makrophyten und Phytoplankton, die mit zunehmender Expositionszeit zu erwarten ist. In Kapitel II wurden neue Wirkmechanismen einer typischen polyphenolischen Allelochemikalie (Tanninsäure) mittels Durchflusszytometrie mit Fluoreszenzmarkern an drei Algenarten evaluiert. Hierbei konnten die Inhibition des Enzymes Esterase und die Produktion reaktiver Sauerstoff-Radikale als neue Anzeiger für allelopathische Effekte detektiert werden. Die nach kurzer Expositionszeit (3 h) und unter natürlich vorkommenden Tanninsäure-Konzentrationen nachgewiesene Inhibition der Esterase-Aktivität stellt eine vielversprechende neue Methodik zur Untersuchung allelopathischer Effekte submerser Makrophyten auf Phytoplankton unter *in situ* Bedingungen dar (Kapitel II).

Die Gründe für unterschiedliche Sensitivitäten verschiedener Phytoplankton-Gruppen und -Arten gegenüber Allelochemikalien sind bisher unbekannt. In der vorliegenden Arbeit wurden der Einfluss Algen-assoziierter Bakterien (Kapitel III) und der Einfluss von Interaktionen zwischen verschiedenen Phytoplanktonarten (Kapitel IV) als potentielle Einflussfaktoren untersucht. Eine Voraussetzung für die Beteiligung assoziierter Bakterien an unterschiedlichen Sensitivitäten ist eine Algenart-spezifische Assoziation. Diese Hypothese wurde durch Vergleiche der Algen-assoziierten Bakteriengemeinschaften vor und nach drastischen Umweltveränderungen der gegenüber Allelochemikalien unsensitiven Grünalge

Desmodesmus armatus und der sensitiven Kieselalge *Stephanodiscus minutulus* getestet (Kapitel III). Sowohl für *D. armatus* als auch für *S. minutulus* wurden artspezifische Bakteriengemeinschaften nachgewiesen (Kapitel III). Allelochemikalien abbauende Bakterien fanden sich jedoch in Assoziation mit beiden Algenarten, so dass deren signifikante Beteiligung an artspezifischen Sensitivitätsunterschieden nicht wahrscheinlich ist (Kapitel III). In Kapitel IV wurde der Einfluss von Interaktionen zwischen Phytoplanktonarten auf deren Sensitivität gegenüber Allelochemikalien am Beispiel der Cyanobakterie *Microcystis aeruginosa* und der Grünalge *D. armatus* untersucht. Hierbei veränderte die Interaktion der beiden Phytoplanktonarten deren Sensitivität gegenüber Allelochemikalien signifikant. Die in einartlichen Kulturen von *M. aeruginosa* nachgewiesene Inhibition der Wachstumsraten durch die allelopathisch aktive Makrophytenart *Myriophyllum spicatum* wandelte sich in Mischkulturen mit *D. armatus* in eine Förderung. Dieses Ergebnis verdeutlicht, dass Resultate aus Experimenten mit einartlichen Kulturen nur bedingt auf die Freilandsituation übertragbar sind (Kapitel IV).

Ein Vergleich der Sensitivitäten des Phytoplanktons gegenüber Allelochemikalien erfolgte bisher nur auf Gruppen-, Gattungs- oder Artniveau. In Kapitel V wurde getestet, ob auch stammspezifische Unterschiede in der Sensitivität auftreten, und ob diese Sensitivitäten aufgrund von Adaption von der Anwesenheit allelopathisch aktiver Makrophyten im Herkunftsgewässer der Stämme abhängen. Um dies zu überprüfen wurden 13 Stämme der Grünalge *Pediastrum duplex* aus drei verschiedenen Makrophyten-freien Gewässern und 10 Stämme aus zwei Gewässern mit allelopathisch aktiven Makrophyten isoliert. Anschließend wurden die Wachstumsraten und die photosynthetische Aktivität aller Stämme unter Einfluss von Allelochemikalien gemessen. Die getesteten Stämme wiesen Unterschiede in der Inhibition ihrer Wachstumsraten und photosynthetischen Aktivität von ca. einer Größenordnung auf. Zukünftige Sensitivitätstests sollten also stammspezifische Unterschiede berücksichtigen, da das Artniveau nicht ausreichend trennscharf bezüglich der Empfindlichkeit gegenüber Allelochemikalien ist. Eine Korrelation von niedrigen Sensitivitäten mit der Herkunft aus Makrophyten-dominierten Gewässern wurde allerdings weder für die Wachstumsraten noch für die photosynthetische Aktivität festgestellt. Somit konnte keine lokale Adaption der Algen an Allelochemikalien nachgewiesen werden.

Summary

Eutrophication led to the loss of the macrophyte-dominated clear-water regime and a shift towards a phytoplankton-dominated turbid regime in many shallow lakes. Allelochemicals released by submerged macrophytes can inhibit the growth of phytoplankton and might therefore contribute to the stabilization of the clear-water regime. This thesis aims to determine the ecological relevance of allelopathic effects between macrophytes and the phytoplankton by detecting new modes of actions of allelochemicals (chapter II) and by evaluating factors that influence the sensitivity of the phytoplankton (chapters III-V).

The significance of allelopathic effects of macrophytes on phytoplankton at the ecosystem scale is still debated. The currently available detection methods have some drawbacks if used for *in situ* investigations as they do not allow a clear separation of allelopathy from other mechanisms. A common problem is competition for nutrients between phytoplankton and macrophytes that is assumed to increase with increasing exposure times. In chapter II new modes of action of the common allelochemical tannic acid were evaluated on three algal species by the use of flow cytometry. The inhibition of esterase activity and the production of reactive oxygen species (ROS) were found as new observation variables. An inhibition of the esterase activity was shown after short exposure times (3 h) and at naturally occurring tannic acid concentrations and is thus a promising tool for future studies on allelopathic effects from submerged macrophytes on phytoplankton under *in situ* conditions (chapter II).

The reasons for sensitivity differences of phytoplankton groups and species to allelochemicals are not yet known. Two factors potentially influencing the sensitivity of the phytoplankton to allelochemicals were tested in this thesis by evaluating the influence of algal-associated bacteria (chapter III), and the impact of interactions between two phytoplankton species on their sensitivity (chapter IV). One prerequisite for bacterial involvement in different sensitivities is their species-specific association to the algae. This hypothesis was tested by comparing the algal-associated bacterial communities of the insensitive green alga *Desmodesmus armatus* and the sensitive diatom *Stephanodiscus minutulus* after drastic changes to the environmental conditions. Both species, *D. armatus* as well as *S. minutulus*, were found to harbor species-specific bacterial communities. However, allelochemical degrading bacteria were associated with both of the tested species, and consequently a bacterial involvement with species-specific sensitivities is not likely (chapter III).

The influence of interactions between phytoplankton species on their sensitivity to allelochemicals was investigated using the cyanobacterium *Microcystis aeruginosa* and the

green alga *D. armatus* (chapter IV). Their interactions significantly altered their sensitivity to allelochemicals. Growth rate inhibition of *M. aeruginosa* by the allelopathically active macrophyte *Myriophyllum spicatum* in single-species cultures changed to an enhancement if co-cultured with *D. armatus.* This finding implies that results of single-species tests may not easily be transferred to the ecosystem level (chapter IV).

Algal sensitivities to stressors have been analyzed at the group, genus or species level. However, sensitivities of algae to allelochemicals may also be strain-specific, and these sensitivities may depend on the presence of allelopathically active macrophytes in their original habitat due to potential adaptations. To test this, 13 strains of the green alga *Pediastrum duplex* were isolated from three different macrophyte-free water bodies, and ten strains from two water bodies containing dense stands of allelopathically active macrophytes. The tested strains exhibited differences of sensitivity to allelochemicals with respect to growth rates and photosynthetic yields of about one order of magnitude. Consequently, future studies on allelochemical sensitivities of algae should also consider strain-specific sensitivities. However, the sensitivities of *P. duplex* growth rates and photosynthetic yields to allelochemicals were not dependent on the presence of allelopathically active macrophytes in their water bodies of origin. Thus, a local adaptation of the target algae to allelochemicals of submerged macrophytes was not shown.

Outline of the thesis

The present thesis is a cumulative work and is based on three peer-reviewed, published articles, plus one article currently under revision. These articles are presented separately as independent chapters with their own introduction, materials and methods, results, discussion and reference part (chapters II – V). The thesis also contains a general introduction (chapter I) and a synthesis that connects my findings to previous works and provides an outlook on questions that remain to be investigated (chapter VI). References of these two chapters are combined and presented subsequent to chapter VI.
The layout of already published manuscripts was modified and Figures and Tables were chronologically numbered throughout the text for consistency.

Chapter II:

Falk Eigemann, Sabine Hilt & Mechthild Schmitt-Jansen. 2013. Flow cytometry as a diagnostic tool for the effects of polyphenolic allelochemicals on phytoplankton. Aquatic Botany 104: 5-14.

Author contributions

FE designed the study, conducted the experiments, analyzed the data, and compiled the manuscript. SH co-wrote the manuscript. MSH co-designed the study and contributed to the text.

Chapter III:

Falk Eigemann, Sabine Hilt, Ivette Salka & Hans-Peter Grossart. 2013. Bacterial community composition associated with freshwater algae: species specificity versus dependency on environmental conditions and source community. FEMS Microbiology Ecology 83: 650-663.

Author contributions

FE designed the study, conducted the experiments, analyzed the data, and compiled the manuscript. SH co-designed the study and co-wrote the manuscript. IS co-analyzed the data. HPG co-analyzed the data and contributed to the text.

Chapter IV:

Xuexiu Chang, **Falk Eigemann** & Sabine Hilt. 2012. Macrophytes support harmful cyanobacteria? Interaction with a green alga reverses inhibiting effects of macrophyte allelochemicals on *Microcystis aeruginosa*. Harmful Algae 19: 76-84.

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Author contributions

XC designed the study, conducted the experiments and analyzed the data. FE co-designed the study, co-conducted the experiments and contributed to the text. SH co-designed the study, co-analyzed the data and compiled the paper.

Chapter V:

Falk Eigemann, Pieter Vanormelingen & Sabine Hilt. Submitted. Sensitivity of the green alga *Pediastrum duplex* Meyen to allelochemicals is strain-specific and not related to co-occurrence with allelopathic macrophytes. PLOS ONE

Author contributions

FE designed the study, conducted the experiments, analyzed the data and compiled the manuscript. PV co-designed the study, co-conducted the experiments and contributed to the text. SH co-designed the study and co-wrote the manuscript.

Chapter I:

General introduction

I.I. Allelopathic effects as potential stabilizing mechanisms of the macrophyte-dominated regime in shallow lakes

Most lakes in the world are small and shallow (Wetzel, 1990). In these lakes, within a certain range of nutrient concentrations, two contrasting regimes exist: The clear-water regime which is dominated by submerged macrophytes and the turbid regime dominated by phytoplankton (Fig. 1; Scheffer et al., 1993; Schmitt and Nixdorf, 1999).

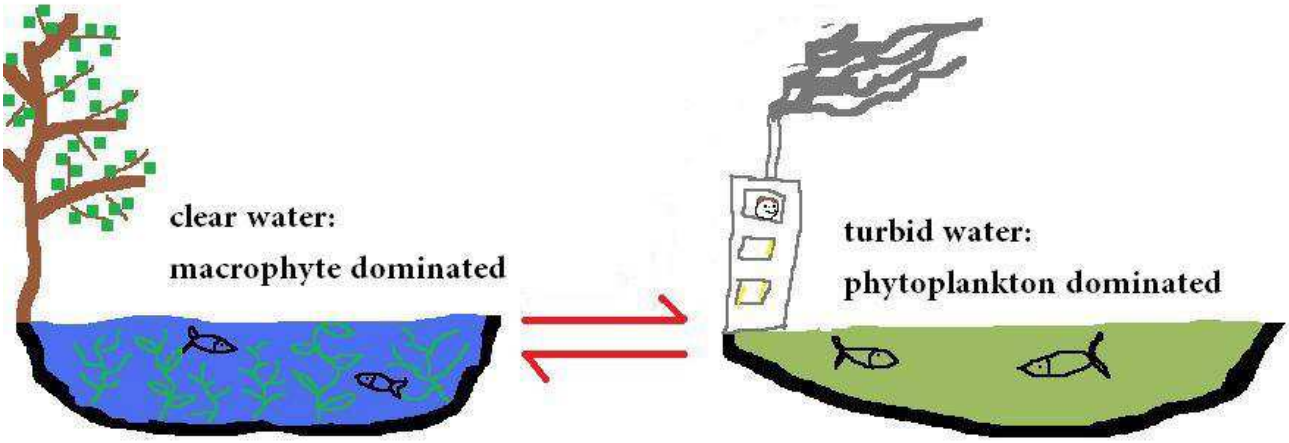

Figure 1:
Alternative regimes in shallow lakes

These two different regimes impose drastic consequences on the biodiversity and productivity of the system (Scheffer, 1998; Hargeby et al., 2004). At low nutrient concentrations only the clear-water regime occurs, whereas at intermediate nutrient levels one of both regimes occurs as a stable state, but above a certain threshold of nutrients only the turbid regime can exist (Scheffer et al., 1993). Various feedback mechanisms stabilize both regimes (Fig. 2; Moss, 1990; Jeppesen et al., 1998; Scheffer and van Nes, 2007). Shifts between both regimes occur at different nutrient loading thresholds, and thus a hysteresis occurs (Scheffer et al., 1993; Scheffer et al., 2001; Jeppesen et al., 2005). After the input of massive nutrient loads due to industrialization and extensive farming in the last century, many shallow lakes switched from the clear-water to the turbid regime (Blindow, 1992; Scheffer et al., 1993). The disappearance

of submerged macrophytes led to decreasing top-down control of phytoplankton by zooplankton, fish-stock changes and toxic cyanobacteria blooms (Wenchuan et al., 2001; Søndergaard et al., 2003). This in turn resulted in severe deterioration of water quality followed by drinking water scarceness, in extreme cases (Qin et al., 2010).

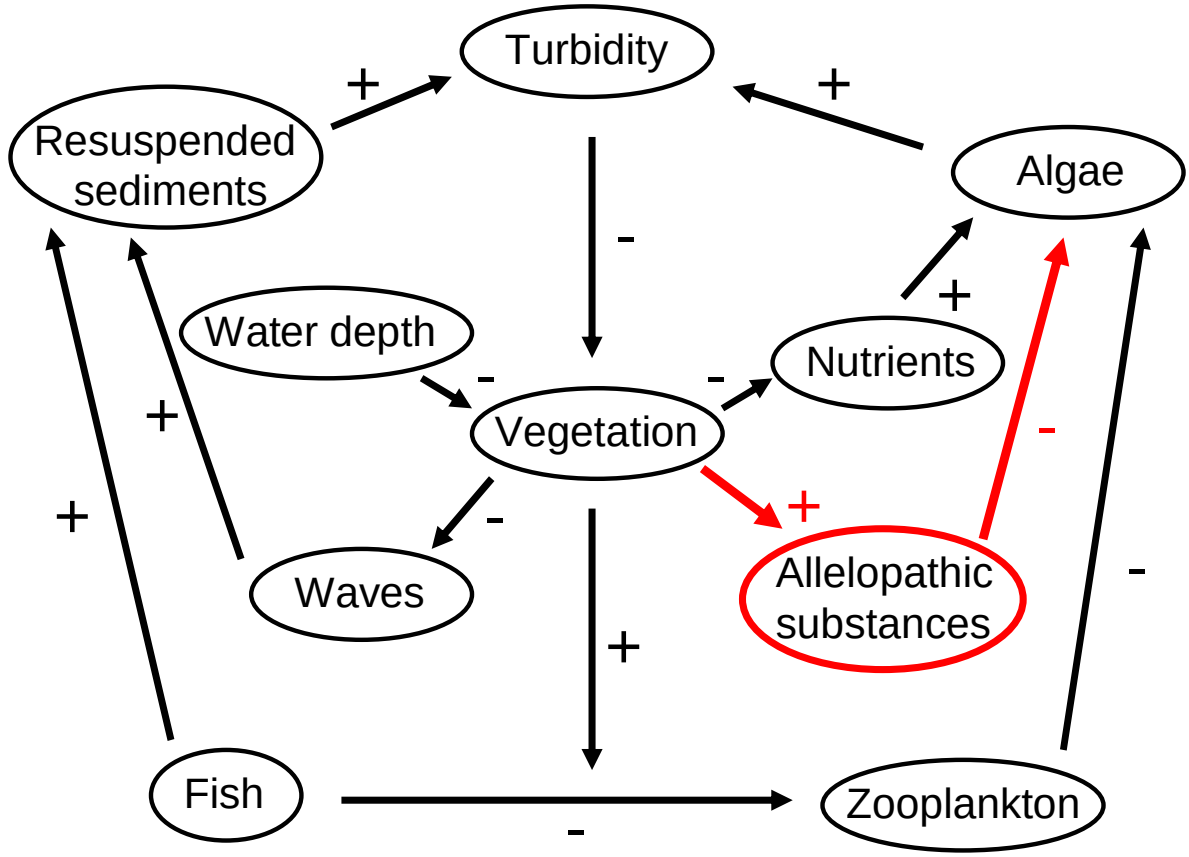

Figure 2:
Factors influencing alternative stable regimes in shallow lakes, modified after Scheffer et al. (1993). Positive impacts are indicated with +, negative with -.

In the last decades much effort has been dedicated to the regeneration of turbid lakes back to their clear-water regime. Reversions, however, are extremely difficult to achieve.

The reduction of nutrient loads requires extensive efforts in terms of time and money, and mostly does not result in rapid improvement of water quality (Scheffer et al., 1993; Hupfer and Hilt, 2008). A sufficient amount of nutrients for phytoplankton and cyanobacteria blooms may be available for several decades as it is enriched in the sediment and resuspension may occur (Søndergaard et al., 2003; Kleeberg et al., 2008). Consequently, a reduction of nutrient

input might be not enough for attaining and maintaining good water qualities in an adequate amount of time (Scheffer et al., 1993).

One factor stabilizing and promoting the switch back to the clear-water regime is the presence of submerged macrophytes (Fig. 2; Scheffer et al., 1993), if macropyhte coverage of the lake reaches a certain threshold (Jeppesen et al., 1994; Lauridson et al., 1996; Blindow et al., 2002; Hilt and Gross, 2008). Submerged macrophytes support the clear-water regime of shallow lakes in manifold ways (Scheffer, 1998): They provide refuge for small fish and phytoplankton grazing zooplankton, they reduce light and nutrient availability for phytoplankton growth, and they increase phytoplankton sinking loss due to decreased perturbation. One additional direct factor of phytoplankton suppression is the release of allelopathically active substances inhibiting phytoplankton growth (Fig. 2; Scheffer et al., 1993; Jeppesen et al., 1998; Mulderij et al., 2007). The relative importance of these factors may vary with lake size and depth, climate, macrophyte abundances and species composition, nutrient concentration (Jeppesen et al., 1999), as well as with seasonality (Blindow et al., 2002). From these various interactions between the macrophytes and the phytoplankton, allelopathic effects earned special attention, because cyanobacteria (one main cause of poor water quality) seemed to be more sensitive to allelochemicals than other phytoplankton groups (Mulderij et al., 2007; Hilt and Gross, 2008).

Allelopathy is defined as the "interaction between plants or microorganisms" (Molisch, 1937), and may thus refer to beneficial as well as detrimental effects. However, in most cases the term allelopathy is used to indicate negative interactions, i.e. if one organism hampers growth and/or development of another organism. Allelopathic effects are triggered by certain secondary metabolites, called allelochemicals, which are released into the environment by a donor organism. Allelopathy is a well understood process in terrestrial systems (see Kruse et al., 2000 for review), but evidence of its effects in aquatic environments at ecosystem level is as yet difficult to establish.

According to Willis (1985), six prerequisites need to be fulfilled to prove the occurrence of allelopathy from aquatic macrophytes on phytoplankton:

(1) a pattern of inhibition of a phytoplankton species by a macrophyte must be shown;

(2) the putative macrophyte must produce a toxin;

(3) there must be a mode of toxin release from the macrophyte into the water column;

(4) there must be a mode of toxin transport and/or accumulation in the environment;

(5) the afflicted phytoplankton species must have some means of toxin uptake; and

(6) the observed pattern of inhibition cannot be explained solely by physical factors or other biotic factors, especially competition and herbivory.

The last point in particular has confounded most studies aiming to prove allelopathy *in situ*, because it is difficult to separate allelopathy from other effects. Interference through competition for light and nutrients is especially difficult to exclude (van Donk and van de Bund, 2002; Gross et al., 2007; Hilt and Gross, 2008). Some studies generally doubt an allelopathic interference among primary producers *in situ* (Forsberg et al., 1990; Glomski et al., 2002) or wonder if resource competition can at all be separated from allelopathic effects (Inderjit and Del Moral, 1997; Lürling et al., 2006). However, numerous laboratory studies have demonstrated an inhibition of phytoplankton growth by macrophyte allelochemicals (e.g., Planas et al., 1981; Wium-Andersen et al., 1983; Aliotta et al., 1992; Gross et al., 1996; Erhard and Gross, 2006; Bauer et al., 2009), and numerous aquatic macrophytes were shown to harbor high amounts of allelochemicals (e.g., Planas et al., 1981; Gross et al., 1996; Bauer et al., 2009). The release of allelochemicals by macrophytes and an accumulation in the environment was also proven by several studies (Nakai et al., 1999; Gross et al., 1996). Due to the frequent occurrence of allelopathically active macrophytes in European shallow lakes, Hilt and Gross (2008) proposed allelopathy as an important mechanism in the interaction of macrophytes and phytoplankton, and allelopathic effects on phytoplankton community patterns in whole lake studies of vegetated, shallow lakes were suggested (Jasser, 1995; Blindow et al., 2002; Lombardo, 2005). Furthermore, the first *in situ* evidence of allelopathic effects of macrophytes on phytoplankton was recently found by Hilt et al. (2006).

In situ relevance, however, of such allelopathic effects is still subject of debate. Often, an interference with competition for macro- and micronutrients between phytoplankton and macrophytes cannot fully be ruled out (Erhard and Gross, 2006; Hilt et al., 2006), i.e. the 6th of Willis' prerequisites can not be fulfilled. Furthermore, allelopathically active compounds are impermanent due to UV-light cleaving and microbial digestion (Glomski et al., 2002; Bauer et al., 2012), and oxygen and redox conditions can influence the stability of allelochemicals (Appel, 1993). The rapid metabolization of allelochemicals after release (Gross, 1999) might be, however, compensated by continuous release (Nakai et al., 1999), and even oxidized and metabolized allelochemicals showed patterns of phytoplankton inhibition (Nakai et al., 2000; Bauer et al., 2012).

Solving the question whether allelopathic effects of macrophytes on phytoplankton significantly contribute to the stabilization of clear-water regimes in shallow lakes thus still requires the investigation of numerous detailed aspects.

I.II. Modes of action of allelochemicals on phytoplankton

The main problem encountered until now in all accomplished *in situ* studies (e.g., Jasser, 1995; Hilt et al., 2006) was the possible interference of allelopathic effects with resource competition. Therefore, measuring methods are required that are not affected by any resource competition to fulfill the 6th of Willis' prerequisites. In the past, most studies of allelopathic reactions on algae have relied solely on growth rates, derived by cell counts or extinction measurements (e.g., Nakai et al., 2000; Mulderij et al., 2003). The disadvantage of these measurements is that a relatively long time is necessary before a reaction of the target organism can be observed, and competition and/or interference with other processes increases with increasing time.

However, allelochemicals are known to act on several processes at the cellular level, i.e. the phytohormonal level, membrane permeability, nutrient uptake, photosynthesis, respiration, enzymatic activities and water relationships (Reigosa et al., 1999). Thereby they tend to affect several physiological processes simultaneously, which means that no process is extremely affected (Reigosa et al., 1999). This makes the *in situ* detection of allelopathy difficult. Summarizing all known modes of action of allelochemicals on phytoplankton is furthermore difficult due to the diverse character of allelochemical molecules (Seigler, 1996; Reigosa et al., 1999). However, for submerged macrophytes and especially *Myriophyllum* spec. polyphenolic allelochemicals were found to be the major inhibiting compounds on phytoplankton (Gross et al., 1996; Leu et al., 2002; Bauer et al., 2009). Besides growth rate inhibition, two specific modes of action of polyphenolic allelochemicals on phytoplankton were described: 1. the inhibition of the photosynthetic yield of the photosystem (PS) II (Körner and Nicklisch, 2002; Leu et al., 2002); and 2. the inhibition of the alkaline phosphatase (APA) (Gross et al., 1996), an exoenzyme used by algae to overcome periods of inorganic phosphorus limitation. However, both modes have some drawbacks when used in *in situ* experiments (see chapter II). For *in situ* investigations and insights into the role of allelopathic effects from macrophytes on phytoplankton at ecosystem level, the discovery of new observation variables is therefore highly desirable. To achieve this, test systems that detect allelopathic effects after a short time exposure and at low, environmentally relevant allelochemical concentrations are necessary.

Flow cytometry is an often used diagnostic technique in ecology to evaluate the metabolic status of cells. It is able to gain data of multiparametric analyses in a short time on the background of fluorometric and light-scatter parameters of single cell populations (Bussaard

et al., 2001). Fluorescence signals can be derived via autofluorescence of the cells or can be mediated by fluorescence markers. Application of flow cytometry to characterize an induced impact of chemicals on microalgae was introduced in the early 2000s (Franqueria et al., 2000; Franklin et al., 2001; Stauber et al., 2002), and was shown to be a suitable tool to detect disturbance of specific cellular algal characteristics (Adler et al., 2007). By now, fluorescent dyes enable statements on specific physiological processes of algal cells and therewith on specific modes of action of chemicals and stressors on algae (Stauber et al., 2002; Adler et al., 2007). In chapter II, I searched for new observation variables of polyphenolic allelochemicals on phytoplankton by using flow cytometry. New observation variables may be applied in *in situ* experiments and possibly accelerate insights into the role of allelopathy from submerged macrophytes on phytoplankton at ecosystem level.

I.III. Factors influencing sensitivities of algae to allelochemicals

For a meaningful evaluation of allelopathic effects *in situ*, it is essential to consider factors that influence the sensitivity of the target phytoplankton. Numerous studies (e.g., Gross et al., 1996; Nakai et al., 1999; Körner and Nicklisch, 2002; Mulderij et al., 2003; Hilt et al., 2006) revealed differences in the sensitivity of different algal species and genera to allelochemicals. Until now however, the reason(s) for different sensitivities of the phytoplankton to allelochemicals are not known.

Müller et al. (2007) were the first to show that distinct bacterial groups isolated from the biofilm and the surrounding water column of the allelopathically active submerged macrophyte *M. spicatum* are able to degrade polyphenolic allelochemicals. This was surprising as polyphenols are known for their anti-bacterial properties (Walenciak et al., 2002), and polyphenol degrading bacteria were only known from anaerobic environments (Mahadevan and Muthukumar, 1980; Schink et al., 2000). Specialized bacteria in the vicinity of allelopathically active submerged macrophytes might be thus one reason for the rapid disappearance of allelochemicals from the water column. *M. spicatum* exudates lost their inhibitory capacity to xenic cyanobacteria and algal cultures over time, also suggesting bacterial degradation (Gross et al., 1996; Nakai et al., 1999).

Algal cells also serve as a bacterial habitat and may therefore harbor specific beneficial bacteria (Bell and Mitchell, 1972). Until now however, most studies on allelopathic effects of macrophytes on algae were carried out either with axenic or xenic algal cultures with an undefined bacterial community. Both treatments do not accommodate the possible effect of

the bacterial community which may metabolize allelochemicals (Müller et al., 2007), transform them biochemically (Scalbert, 1991), build mechanical barriers such as extracellular polysaccharides (Decho, 1990) or otherwise interact with the phytoplankton species via symbiosis, commensalism or antagonism (Cole, 1982; Grossart, 1999).

Consequently, bacteria attached to the submerged macrophytes or the target algae as well as bacteria suspended in the surrounding water column may have an impact on allelopathic interactions by inactivation or modulation of allelopathically active substances. Bauer et al. (2010) pointed to a bacterial interference of algal sensitivities, as xenic and axenic cultures of the green alga *Desmodesmus armatus* and the diatom *Stephanodicus minutulus* showed significantly different sensitivity to the polyphenol tannic acid (TA) in laboratory experiments. Associations with bacteria that are able to degrade allelochemicals may thus be one explanation as to why certain algal species are less sensitive to allelochemicals. However, a prerequisite for a bacterial involvement in different sensitivities is a species-specific association. If environmental conditions would determine bacterial associations to the algae, the role of the bacteria on algal sensitivities would be negligible, because different algal species in the same habitat would share the same bacterial community. In chapter III, I examined bacterial associations with two freshwater algal species with contrasting sensitivities to allelochemicals under various environmental conditions and bacterial source communities to test for species-specific associations, and tried to relate the appearance of allelochemical degrading bacterial phylotypes to algal sensitivities.

Even though sensitivity was mostly proven to be species-specific (e.g., Körner and Nicklisch, 2002), some general patterns of sensitivity of phytoplankton groups were found (e.g., Jasser, 1995; Körner and Nicklisch, 2002; Hilt and Gross, 2008). Green algae appeared to be less sensitive to allelochemicals than diatoms and cyanobacteria (Hilt and Gross, 2008). Most of the accomplished studies used single algal cultures and neglect the *in situ* conditions, where several phytoplankton groups and species co-exist, and where interactions between the targets of the macrophyte allelochemicals will consequently occur. However, also under *in situ*-like conditions, allelopathic effects of macrophytes on a natural phytoplankton community supported green algae, whereas cyanobacteria were inhibited (Jasser, 1995). These higher sensitivities of cyanobacteria promoted the idea of using allelopathic effects as an effective measure for the control of undesired cyanobacterial blooms, especially in highly eutrophic environments. However, it is still not known how sensitivities are affected by interactions between phytoplankton groups and/or species. In ecotoxicological studies, single-species algal cultures reacted differently compared to communities of different algal groups and species

(Schmitt-Jansen and Altenburger, 2008). Thus, it is doubtful that single-species tests reflect the outcome of allelopathic interactions at ecosystem level. Before allelopathically active macrophytes are applied as intervention measures for toxic algal blooms, more realistic studies are needed in order to gain information if an application of allelochemicals is reasonable for controlling harmful cyanobacteria. In chapter IV, I investigated the impact of an interaction between a cyanobacterium and a green alga on their sensitivities to allelochemicals. The results of this part of my project may contribute to reasonable applications of allelopathic macrophytes or allelochemicals as measures against toxic cyanobacterial blooms.

Phytoplankton species comprise numerous different strains that exhibit partly differing physiologies (Lakeman et al., 2009). Therefore, considering different sensitivities of algal groups and species, the question arises if different algal strains within a species also reveal differing sensitivities to macrophyte allelochemicals. Different phytoplankton strains of one species do exhibit deviating characteristics to specific traits (e.g., Fisher et al., 1973; Murphy and Belastock, 1980), including sensitivity differences towards stressors with a magnitude of up to several orders (Jensen et al., 1974; Behra et al., 1999). Low sensitivities were mostly correlated with a permanent or long-time exposure to the stressor, and accordingly reported as adaptations (Fisher et al., 1973; Murphy and Bellastock, 1980). In the case of aquatic allelopathy, epiphytic algal species were found to react less sensitively than planktonic species (Hilt, 2006; Hilt and Gross, 2008). At first glance this seems counter-intuitive, since epiphytic species live adjacent to the donor of the allelochemicals. Furthermore, one might expect them to contribute more to the reduction of light to the macrophyte than planktonic species which results in a higher pressure for the macrophyte. Epiphytic species, however, might have undergone co-evolution and built adaptations against allelochemicals from macrophytes originating from the same habitat (Reigosa et al., 1999). As *argumentum e contrario* this may explain the success of certain invasive, allelopathically active species because their targets do not exhibit any kind of adaptation against their allelochemicals. This theory was manifested with the "novel-weapon-hypothesis" (Callaway and Aschehoug, 2000; Bais et al., 2003).

So far, only one study on allelopathy focused on algal strain sensitivities with respect to their origin. Al Sheri (2010) was able to show that a *Scenedesmus obliquus* strain isolated from a macrophyte-free pond revealed higher sensitivities to allelochemicals compared to a strain from a pond containing the allelopathic macrophyte *Stratiotes aloides*. In this study, however, only one strain from each origin was tested, which is not a sufficient number with respect to

final conclusions on adaptation processes to allelochemicals in a broader context. In chapter V, I examined 13 strains of the same green algal species from macrophyte-free water bodies and ten strains from water bodies hosting dense stands of allelopathically active macrophytes for differences in their sensitivities to allelochemicals, and related these sensitivities to their origin. Results of this study may have important implications for a generalistic classification of allelopathic interactions with invasive species and clarify if sensitivities are indeed a species-specific criterion.

Besides biotic factors like interactions between acceptor species and/or strains and bacterial associations, abiotic factors were also shown to influence outcomes of sensitivity rankings of the phytoplankton to allelochemicals. Amongst others, light quantity and quality (Bauer et al., 2012), applied parameter and method (Hilt et al., 2012), and the mode of allelochemical addition (Reigosa et al., 1999; Hilt et al., 2012) were shown to influence sensitivities of the phytoplankton. Evaluation of the specific contributions to the overall sensitivity of these factors was therefore a further aim of this thesis and is discussed in chapter VI.

I.IV. Aims of the project

This thesis aims at determining and evaluating different factors influencing the sensitivity of phytoplankton to allelochemicals, and at the detection of additional observation variables for allelopathic effects. Achievements in these sectors of research will contribute to a better understanding of allelopathic effects of submerged macrophytes on phytoplankton, and thus enable a better appraisal of ecosystem relevance of allelopathic effects in aquatic environments. For my studies, I chose model organisms that were already used in antecedent studies on aquatic allelopathy, allowing better comparisons of my results to the existing body of knowledge. The specific aims of the realized work-packages were:

Chapter II: The goal of this chapter was to find new observation variables for allelopathic effects of polyphenolic allelochemicals on phytoplankton by means of flow cytometry. Additional variables may facilitate predictions of the *in situ* relevance of allelopathic interactions on phytoplankton. Therefore, I tested whether esterase activity, membrane integrity and the production of reactive oxygen species (ROS) were influenced by the polyphenolic allelochemical tannic acid (TA) with specific fluorescent markers. To enable general conclusions, examinations were conducted with three different algal species and after different exposure times.

Chapter III aimed at testing whether bacteria are species-specifically associated to freshwater algae. This hypothesis was tested by changing the environmental conditions and/or bacterial source communities. Therefore, the algal´s bacterial community compositions (BCCs) before and after *in situ* incubation of initially axenic and xenic algal cultures in a lake and the algal´s BCCs before and after changes in the bacterial source communities of the algal cultures were compared. Furthermore, algal sensitivities to allelochemicals and the bacterial phylotypes associated with the algae were evaluated in order to gain information to which extent bacterial communities play a role in the different sensitivities of algal species to allelochemicals. For that reason, two freshwater algal species with a contrasting sensitivity to allelochemicals were used.

Chapter IV: The main goal of this chapter was to determine if interactions between two phytoplankton species alter their sensitivity to allelochemicals. I hypothesized that *Microcystis aeruginosa*, a common and rather sensitive cyanobacterium in single-species cultures will also be suppressed in a mixed culture with the addition of the rather insensitive green alga *Desmodesmus armatus*. To test this I measured the effect of allelochemicals on single-species and mixed cultures of these species by the addition of the allelochemical TA and in co-existence experiments with the allelopathically active *Myriophyllum spicatum*.

Chapter V: Within this chapter, I aimed at testing whether different strains of one phytoplankton species exhibit different sensitivities to allelochemicals. The chapter further aimed at testing whether adaptations to allelochemicals occur, i.e. if sensitivities of strains isolated from ponds with *Myriophyllum* spec. are lower than those of strains from macrophyte-free ponds. To test for this, altogether 23 algal strains of the green alga *Pediastrum duplex* were isolated from three macrophyte-free water bodies and two water bodies with stands of allelopathically active macrophytes (*Myriophyllum* spec.). Afterwards, I compared the sensitivities of the strains to polyphenolic allelochemicals after single additions of TA and in co-existence experiments with *M. spicatum*, and related the sensitivities with the origin of the strains.

Chapter II:

Flow cytometry as a diagnostic tool for the effects of polyphenolic allelochemicals on phytoplankton

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Abstract

We investigated the impact of the polyphenol tannic acid (TA) on the green algae *Desmodesmus armatus* and *Scenedesmus vacuolatus* and the diatom *Stephanodiscus minutulus* in order to find new diagnostic tools for allelopathic effects on phytoplankton. Esterase activity, membrane integrity and production of reactive oxygen species (ROS) were tested using flow cytometry with specific fluorescent markers. For comparison, growth rate and photosynthesis, two variables known to be affected by TA, were evaluated. Algae were exposed to TA concentrations between 0.6 and 30 µmol L^{-1} for 3, 14 and 24 hours. A significant inhibition of esterase activity was detected at every time point in all three tested algal species at 30 µmol L^{-1} TA and in most other treatments when TA concentrations exceeded 3 µmol L^{-1}. A significant production of ROS could also be detected in all three algal species, but only after a longer exposure period. Changes in membrane rigidity revealed no consistent patterns of enhancement or inhibition when tested with different TA concentrations, algal species and exposure time. Growth rates of all algae were significantly inhibited after 24 h, whereas *D. armatus* was the only species for which the photosynthetic yield did not decline.

The effects on esterase activity and ROS production indicate a general influence of polyphenolic allelochemicals on phytoplankton, but also reveal patterns which vary between species, concentrations and exposure times. Changes in esterase activity were the most sensitive variable, and could be detected after short exposure periods and at naturally occurring concentrations. Thus, esterase activity may be a suitable variable for future investigations into the allelopathic effects of submerged macrophytes on phytoplankton.

Introduction

Allelochemicals exuded from submerged macrophytes may inhibit other aquatic primary producers such as phytoplankton or epiphyton, providing macrophytes with a competitive advantage for light and nutrients. Numerous aquatic macrophytes contain and release allelochemicals into the ambient water body (Gross et al., 1996). Most studies into the effects of allelochemicals on phytoplankton have used plant extracts or purified plant compounds, even though such procedures fail to reflect natural lake conditions (Hilt and Gross, 2008). Coexistence experiments placing phytoplankton (typically in dialysis bags) among macrophytes are closer to *in situ* conditions, but often suffer from interfering processes that complicate the isolation of allelopathy as a primary mechanism. One of the major confounding factors is the potential simultaneous competition for nutrients between macrophytes and phytoplankton (Inderjit and Del Moral, 1997; Hilt et al., 2006; Gross et al., 2007). This could be prevented by short-term experiments. Such experiments, however, would require a sensitive observation variable that detects a phytoplankton response to allelochemicals within a short exposure period.

A few specific modes of action on phytoplankton have been identified for some of the known aquatic allelochemicals (e.g., tellimagrandin II (Gross et al., 1996; Leu et al., 2002) and ethyl 2-methyl acetoacetate (Hong et al., 2008)). Polyphenols, a common and well investigated class of allelopathically active compounds in submerged macrophytes of the genus *Myriophyllum* (Gross et al., 1996; Gross, 2003; Bauer et al., 2009), have been shown to inhibit two processes in algae: 1) alkaline phosphatase (APA) activity (Gross et al., 1996) and 2) photosystem II (PS II) activity (Körner and Nicklisch, 2002; Leu et al., 2002). Both processes, however, have some drawbacks when used in coexistence experiments. The exoenzyme APA is only produced during periods of inorganic phosphorus limitation, and effects on PS II activity are also influenced by nutrient limitation (Lippemeier et al., 2003). In addition, significant inhibitions of APA and PS II were only found after at least 3 days of exposure. Studies on other modes of action that respond more rapidly are lacking.

A second important aspect that requires the detection of various observation variables is the different sensitivity of phytoplankton groups and species towards allelochemicals. Chlorophytes generally seem to be less sensitive than diatoms and cyanobacteria (Hilt and Gross, 2008) in terms of growth and photosynthetic inhibition (Körner and Nicklisch, 2002), and epiphytes appear to be less sensitive than planktonic species (Hilt, 2006). Whether this holds true for other variables is not yet known. A first comparison of different methods (co-incubation with and without macrophytes *in situ* and in aquaria, with or without tannic acid addition) and variables (fluorescence-based chlorophyll a concentrations, PS II activity, cell counts, or biovolume) used

to detect the allelopathic effects of macrophytes on two green algal species revealed significant differences between variables (Hilt et al., 2012).

Despite recent advances in this field of study, the molecular interaction between allelochemicals and possible cellular targets remains unclear. Every organic chemical exhibits a non-specific or baseline toxicity to an organism, due to the fact that chemicals penetrate biological membranes according to their lipophilicity. As this process is driven by partitioning between phases, baseline toxicity correlates with the K_{ow} (octanol-water partition coefficient) of a substance and thus represents the minimum toxicity of a given substance towards an organism. These empirical relationships can be modelled (e.g., Altenburger et al., 2004) and used to predict the minimum toxicity of untested substances. More specific interactions, such as binding to enzymes or receptors, typically result in a higher toxicity than the baseline toxicity. A comparison of the effect levels between different observation variables and baseline toxicity may indicate the specificity of a given variable. There are, however, prerequisites for the applicability of these models. For instance, the molecular structure of an allelopathically active substance must be known, thus confining most studies to natural products. Another problem arises due to the complexity of the known polyphenolic allelochemicals (Gross et al., 1996). All models that have estimated the effects of chemicals on algae (e.g., Altenburger et al., 2004) have used well-defined molecules with low molecular weights. For natural products with high molecular weights, it is unlikely that the existing models are suitable. Natural products are difficult to characterize and often highly degradable (Müller et al., 2007; Bauer et al., 2012), thus complicating analyses. One further aim of this study was therefore to consider the applicability of available baseline toxicity models to polyphenols.

The diagnostic technique of flow cytometry can be used to evaluate the metabolic status of cells, and was initially shown to be a suitable tool for detecting the disturbance of specific cellular algal characteristics by metals (Franklin et al., 2001; Stauber et al., 2002) and paraquat (Franqueria et al., 2000). Fluorescence signals can be derived via direct auto-fluorescence measurements of the cells, or can be mediated after staining with suitable fluorescence markers. In the green alga *Scenedesmus vacuolatus*, fluorescence markers for membrane permeability and potential, as well as mitochondrial respiration and esterase activity were used to investigate the effects of various xenobiotics (Adler et al., 2007). In addition, fluorescence markers for the production of reactive oxygen species (ROS) were established (Le Bel et al., 1992) and used in flow cytometric approaches with algae (Szivak et al., 2009). Hong et al. (2008) were the first to test whether an allelochemical, ethyl 2-methyl acetoacetate (EMA) produced by reeds, inhibits processes in algae; namely esterase activity and ROS-production with established fluorescence

markers. *Microcystis aeruginosa* cultures exposed to the EMA, however, revealed either enhanced or decreased enzyme activity depending primarily on exposure time (Hong et al., 2008). The same was found to hold true for esterase activity in *M. aeruginosa* and *Selenastrum capricornutum* cultures exposed to acid mine drainage water (Regel et al., 2002). Furthermore, an enhanced ROS production was detected in phytoplankton and cyanobacteria cultures exposed to metals and EMA, and has been interpreted as a consequence of the inhibition of detoxification enzymes such as esterases (Szivak et al., 2009) or as acute cell damage (Hong et al., 2008). In the latter case, even a subsequent increase in detoxification enzymes such as esterases was proposed (Hong et al., 2008). Still, the enhanced production of ROS may be accepted as a general early response of algae to a stressor (Szivak et al., 2009).

Polyphenols are able to penetrate cell membranes due to their amphiphilic or lipophilic structure (Leu et al., 2002). Possible impacts on cell membrane integrity in algal cells due to polyphenols might therefore be detectable with fluorescence dyes established for other phytotoxicants (Franklin et al., 2001; Adler et al., 2007). Gram-negative bacteria exposed to polyphenols increased their membrane permeability (Yi et al., 2010), whereas *in vitro* studies revealed that the polyphenol tannic acid aggregated phospholipid bilayers, thus reducing the fluid spacing between them (Simon et al., 1994). At higher concentrations of tannic acid, however, phospholipid bilayers became unstable (Simon et al., 1994). Polyphenols can, depending on redox conditions (primarily oxygen availability and pH), be oxidized and bind to other metabolites by hydrogen bonding and hydrophobic interactions, thus acting as potential enzyme inhibitors (Gross et al., 1996; He et al., 2006). However, studies on these possible effects of polyphenols on phytoplankton are lacking.

The aims of the present study were to investigate (I) whether new effect variables of polyphenols on phytoplankton can be detected by the use of flow cytometry, and (II) whether these effects can be detected after short-term exposure and at naturally occurring allelochemical concentrations. We therefore modelled EC_{50} values for TA on three algal species after measuring changes in membrane integrity, production of ROS and esterase activity with specific fluorescence markers. Results were compared to the inhibition of growth rates and photosynthetic activity.

Materials and Methods

Test organisms and culture conditions

A synchronized, unicellular non-axenic culture of the green alga *Scenedesmus vacuolatus* Shihira et Krauss (strain 211-15; SAG University of Göttingen, Germany) was photoautotrophically grown in 2-fold Gimme-Bordman medium (pH 7.2) at 28 ± 0.5°C under 14:10 h light:dark conditions at 370 µmol photons m^{-2} s^{-1} (Altenburger et al., 2004). The non-axenic green alga *Desmodesmus armatus* Chodat (SAG University of Göttingen, Germany) and the diatom *Stephanodiscus minutulus* Kütz (Kleve et Möller) (SAG University of Göttingen, Germany) were grown in modified MIII medium (Nicklisch, 1992) at pH 7.5 – 7.9 at 20 ± 0.5°C at 80 µmol photons m^{-2} s^{-1} under 12:12 h light:dark conditions in a conditioning cabinet. The MIII medium contained $CaSO_4$ 0.5 mM, $CaCl_2$ 0.5 mM, $MgSO_4$ 0.25 mM, $NaNO_3$ 0.5 mM, KH_2PO_4 0.05 mM, KCl 0.1 mM, Na_2SIO_3 0.4 mM, HCl 0.75 mM, $NaHCO_3$ 2 mM, $FeCl_3$ 0.01 mM, Na_2 EDTA 0.02 mM, trace elements H_3BO_3 4 µM, $MnSO_4$ 0.8 µM, $ZnSO_4$ 0.08 µM, Na_2MoO_4 0.04 µM, $CuSO_4$ 0.04 µM, $AlK(SO_4)_2$ 0.08 µM, $CoCl_2$ 0.04 µM, $NiSO_4$ 0.04 µM, KBr 0.08 µM, KJ 0.04 µM and H_2SO_3 0.06 µM. Both cultures were shaken gently at 60 rpm. All algal cultures grew exponentially when applied in the experiments.

Inhibition tests with tannic acid (TA)

Solutions of the hydrolysable polyphenol TA (Fluka, filling code: 403955/1 64400) were freshly prepared for each experiment with the respective media used for algal maintenance. A 33 µmol L^{-1} stock-solution was diluted with medium to final concentrations of 0.6, 3, 6, 12 and 30 µmol L^{-1}. Exposure times were 3, 14 and 24 h, always starting approximately with the light periods. Fourteen hour exposures of *D. armatus* and *S. minutulus* were carried out exclusively in light conditions. Each experiment was conducted with 3–5 replicates. For each replicate, 800 µL of an algal suspension were added to 7.2 mL of a TA solution (or medium, as a control) and 20 µL of a carbonate buffer (with a final concentration of 1.5 mmol L^{-1} $NaHCO_3$). The pH value for the Gimme-Bordman medium was monitored during exposure without showing differences after 24 h of exposure to the highest TA concentration (Control: pH 7.23 ± 0.15; 30 µmol L^{-1} TA: pH 7.1 ± 0.17, Mann-Whitney-U (MWU) test, $p = 0.487$). Initial algal cell concentrations ranged between 30,000 and 100,000 cells mL^{-1}. Test conditions were identical to growth conditions, except for the use of 10 mL Pyrex – vials (Pyrex culture tubes, QVF Glastechnik GmbH, Wiesbaden, Germany) instead of 100 mL Erlenmeyer flasks, and stirring by a magnetic bar (30 s stirring at 200 rpm, 3 min 30 s off) instead of shaking.

Stability of TA

To assess the stability of TA, stock solutions (33 µmol) with and without the addition of an algal suspension (final concentration ca. 100,000 cells mL^{-1}) were tested by the Folin-Ciocalteau method (Lowry et al., 1951). Solutions containing algae were filtered prior to measurement with a 50 mL syringe (Omnifix Luer Lock solo B/Braun, Melsungen, Germany) equipped with a 0.2 µm membrane filter top (Spartan 13/0.2 RC, Whatman plc, Kent, United Kingdom). 0.1 mL of solution were abstracted after 24 h of algal exposure and mixed with 0.75 mL Na_2CO_3 solution (217 g L^{-1}) and 0.25 mL Folin reagent (Sigma-Aldrich, Munich, Germany) and incubated for 30 minutes at room temperature. After using freshly prepared TA for the calibration curve, total phenolic compounds (TPC) were measured with a spectrophotometer (UVIKON 923 UV/VIS, Rossdorf, Germany) at a 750 nm wavelength. Measurements do not only account for TA, since the Folin-Ciocalteau method also detects other phenolic compounds and proteins. As we did not perform correction measurements, protein excretions by the algae might have lead to an overestimation of the phenolic compounds, and thus we were estimating the overall reducing capacity (Appel et al., 2001). Furthermore, TA can be photolytically cleaved, and it is thus possible that phenolic cleavage products may have been measured as well. However, cleavage products of TA also influence algal growth (Bauer et al., 2012) and thus the Folin-Ciocalteau method provided a rough estimate of the active phenolic compounds.

Without the addition of algae, the polyphenol concentration increased by about 0.5%, whereas the concentration decreased by 10% in algal solutions within 24 h of exposure, though this decrease does not account for TA attached to or taken up by algal cells. Due to this minor change in concentrations, the active concentration we refer to in all experiments is regarded to be comparable to the nominal TA concentration.

Cell staining and fluorescence markers

We tested the influence of TA on esterase activity by fluorescein diacetate staining (FDA, Table 1, purchased from Fluka (Seelze, Germany)). Propidium iodide (PI, a marker for the possible disturbance of membrane integrity) and hydroethidium (HE, a marker for the production of ROS) were purchased from Sigma (Karlsruhe, Germany) (Table 1). Each fluorescence marker was optimized in terms of the concentration and duration of labelling (Table 1) according to clear peaks in the detection range of the respective fluorescence detector of the flow cytometer. The three algal stains were used separately for each experiment.

Test for suitability of HE as a marker for ROS production

Due to the instability of hydroethidium (HE, Szivak et al., 2009), recent studies used difluorodihydrofluorescein diacetate (H_2DFFDA) as an ROS marker. However, esterases need to cleave the H_2 DFFDA molecule inside the cell before it is converted into a fluorescence product by ROS. H_2 DFFDA was therefore not a suitable marker for our investigations, as we found decreased esterase activity in our experiments. To test the suitability of HE as a marker, ROS production was induced with a tert-butyl hydro-peroxide (90.12 g mol^{-1}, TBHP, Sigma-Aldrich, Seelze, Germany) solution in all three algal species, and measured with a flow cytometer with the HE marker (described below). The induction of ROS by TBHP resulted in a concentration-dependent increase in ROS levels in all three algal species (data not shown). HE thus appears to be a valid marker for ROS in our study.

Table 1: List of applied fluorescence markers

Marker	**Indicator of**	**Concentration of working solution (mM)**	**Quantity used for 600 µL sample (µL)**	**Incubation time (min)**	**Staining: active cells**	**Staining: Inactive cells**	**Mechanism of staining**
FDA (fluorescein diacetate)	Esterase activity/ disturbance of cell membrane	0.024	9	20	+		Inside the cell esterases cleave the FDA molecule into a hydrophilic fluorescent product.
PI (Propidium iodide)	Membrane integrity	15	0.6	20		+	Intercalates with nucleic acids. Unable to pass intact membranes.
HE (Hydroethidium)	Production of ROS	5	0.6	10		+	Intracellular peroxidases and ROS convert HE to ethidium.

Flow cytometry

Flow cytometric measurements were conducted with a FACS Calibur apparatus (Becton Dickinson, Heidelberg, Germany). The set-up parameters were optimized for *S. vacuolatus* cells (Voltage: Fl1: 600, Fl2: 550, Fl3: 400; Amplification gain: 1 for all detectors; measured in the log-mode) and applied for *S. minutulus* and *D. armatus* cells with minor changes (Fl3 voltage was increased to 600 for *S. minutulus*, and Fl3 voltage changed to 300, Fl1 to 500 and Fl2 to 400 for *D. armatus* cells). To obtain metabolically inactive cells as negative controls, cells which had not been exposed to TA were boiled for 30 min at 90°C. Each 600 µL sample was inserted into a 10 mL plastic vial, placed into the flow cytometer, and following a forerun of 30 seconds, 5,000 cells were measured.

For corrections, each sample was measured prior to staining in order to obtain fluorescence values for the respective detector without a fluorescence marker. These values were subtracted from the values gained from the subsequent measurement with a fluorescence marker. Furthermore, all obtained peaks were gated in order to discriminate fluorescence which was not derived from the stained cells. For later calculations, the gained fluorescence values (gated median) of the respective detector were used. Differences between treatments and controls were expressed as a percentage difference in the fluorescence values. Thereby each fluorescence value of the treatment (gated median) was compared with the mean (of gated medians of the replicates) of the controls. Means of the percentage inhibition of the treatments were subsequently calculated.

Interference of fluorescence markers with TA

Fluorescence overlapping of TA and fluorescence markers in the range of the particular detector was tested by a photospectrometer (UVIKON 923 UV/VIS, Rossdorf, Germany) at wavelengths between 190 and 900 nm. Each stain was mixed with a TA stock solution at the same concentration used for the measurements. No crossover of TA and fluorescence markers in the respective detection range of the flow cytometer could be detected.

Cell counts

For cell counts, 500 µL of algal suspension were added to 5 mL Casyton and measured in duplication with a CASY (Schärfe Systems, Reutlingen, Germany) particle counter using default parameters with a dilution factor of 11. Measurements were gated in order to discriminate non-algal particles. Growth rates µ were calculated as:

$\mu\ (d^{-1}) = \ln(\text{number of cells } (t_i) - \text{number of cells } (t_0)) / t_i$

where t_i is time in days. Inhibitions of growth rates were only detectable after 24 h of exposure to TA for technical reasons (synchronisation of *S. vacuolatus* after 24 h, and longer generation times in the other two algal species).

Photosynthetic yield

Photosynthetic yields were quantified by pulse-amplitude-modulated (PAM) fluorometry, measuring the maximum quantum yield (Imaging-Maxi-PAM and Phyto-PAM, Fa. Walz, Effeltrich, Germany). *D. armatus* and *S. vacuolatus* cells were measured with the Imaging-Maxi-PAM. Two mL of algal suspension were placed into a 24-well plate (TPP, Trasadingen, Switzerland) and dark-adapted for 5 min. The maximum quantum yield was determined with the variable fluorescence at 665 nm (Set-up: measuring light intensity: 3, frequency: 1, damping: 2, PAR: 0) (Schreiber, 1996) and assessed by applying a single saturation pulse (Franz et al., 2008). Because the diatom *S. minutulus* is excited at different wavelengths, cultures were measured with the four-wavelength-excitation Phyto-PAM fluorometer. Algal suspensions were dark adapted for 15 min and subsequently 2 mL of algal suspension were placed in a cuvette equipped with a magnetic bar and a stamp. The maximum quantum yield was determined after 3 min of illumination with a measuring light (Set-up: measuring frequency: 2, damping: 3) by applying a single saturation pulse (Körner and Nicklisch, 2002).

Calculation of inhibition and concentration-response fitting

All treatments were compared to the means of controls, and inhibition was calculated as

$$\text{inhibition (\%)} = (T_c - T_t)/T_c * 100$$

where T_c is the mean of the controls and T_t is the mean treatment value.

Concentration-response curves were modelled by fitting the data with the log-logistic equation

$$y = A_2 + (A_1 - A_2)/(1 + (X/X_0)^{\alpha})$$

where y is the inhibition (in %), A_1 is the maximum response, A_2 is the minimum response, X is the concentration, X_o is the concentration at median efficacy, and α represents the slope. When the response curves approached a value of 0 for A_1 and/or 100 for A_2, they were fixed to 0 and/or 100, respectively. EC_{20} and EC_{50} values correspond to the concentrations at which 20% and 50% inhibition occur, and were calculated by rearranging the equation to:

$$X = (((A_1 - A_2)/(y - A_2) - 1) * (X_0^{\alpha}))^{1/\alpha}$$

with y set to 20 and 50, respectively.

All analyses were performed using the software Origin (Microcal, Software, Northhampton, MA, USA).

Statistical analyses

The treatments were compared with the respective controls using the corrected fluorescence values and Mann-Whitney-U tests. Treatments were considered to be significantly different with $p \leq 0.05$. Correlation coefficients between treatment values and TA concentrations were calculated with Spearman`s rho analysis. All statistical analyses were performed using the software package Pasw17 (SPSS).

Modeling baseline toxicity of TA on algae

The model ChemProp (UFZ Department of Ecological Chemistry 2011. ChemProp 5.2.4. http://www.ufz.de/index.php?en=6738) was used to estimate the log K_{ow} values of TA, using the formula $C_{76}H_{52}O_{46}$ with a molar weight of 1701.2 g. Subsequently, the EC_{50} value of TA on algae was calculated with the equation:

$\log EC_{50}\,\text{mol}\,L^{-1} = -0.864 * \log P - 0.9$,

where P is the modelled K_{ow} value (Altenburger et al., 2004).

Results

Inhibition of esterase activity

Esterase activity was significantly inhibited (from 20 to 90%) at 30 µmol L^{-1} TA for every exposure period and algal species (Fig. 3A-C). Esterase activity significantly increased for *S. minutulus* and *S. vacuolatus* after a 24 h exposure at lower TA concentrations (Fig. 3B-C). Absolute fluorescence values of the controls and inactivated controls are given as supplementary material (Table S1, subsequent to this chapter). Several effect concentrations could not be calculated due to inappropriate data (Table 2).

Esterase activity in *D. armatus* was inhibited up to 80% after a 24 h exposure from 6 µmol L^{-1} TA onwards (Fig. 3A). The 14 h exposure treatment of *D. armatus* revealed a V-shaped concentration-response curve with an inflexion point at 6 µmol L^{-1} TA (Fig. 3A). The EC_{20} was more than 50-fold higher after a 3 h exposure compared to a 24 h exposure (Table 2).

In *S. minutulus*, esterase activity was inhibited by TA concentrations greater than 3 µmol L^{-1} after 3 and 14 h exposures. After a 24 h exposure, esterase activity was enhanced up to 80% at 3 µmol L^{-1}, resulting in a V-shaped concentration-response curve (Fig. 3B). EC_{20} values were comparable for 3 and 14 h exposure times (3 and 2.5 µmol L^{-1}), but higher (27 µmol L^{-1}) after a 24 h exposure (Table 2).

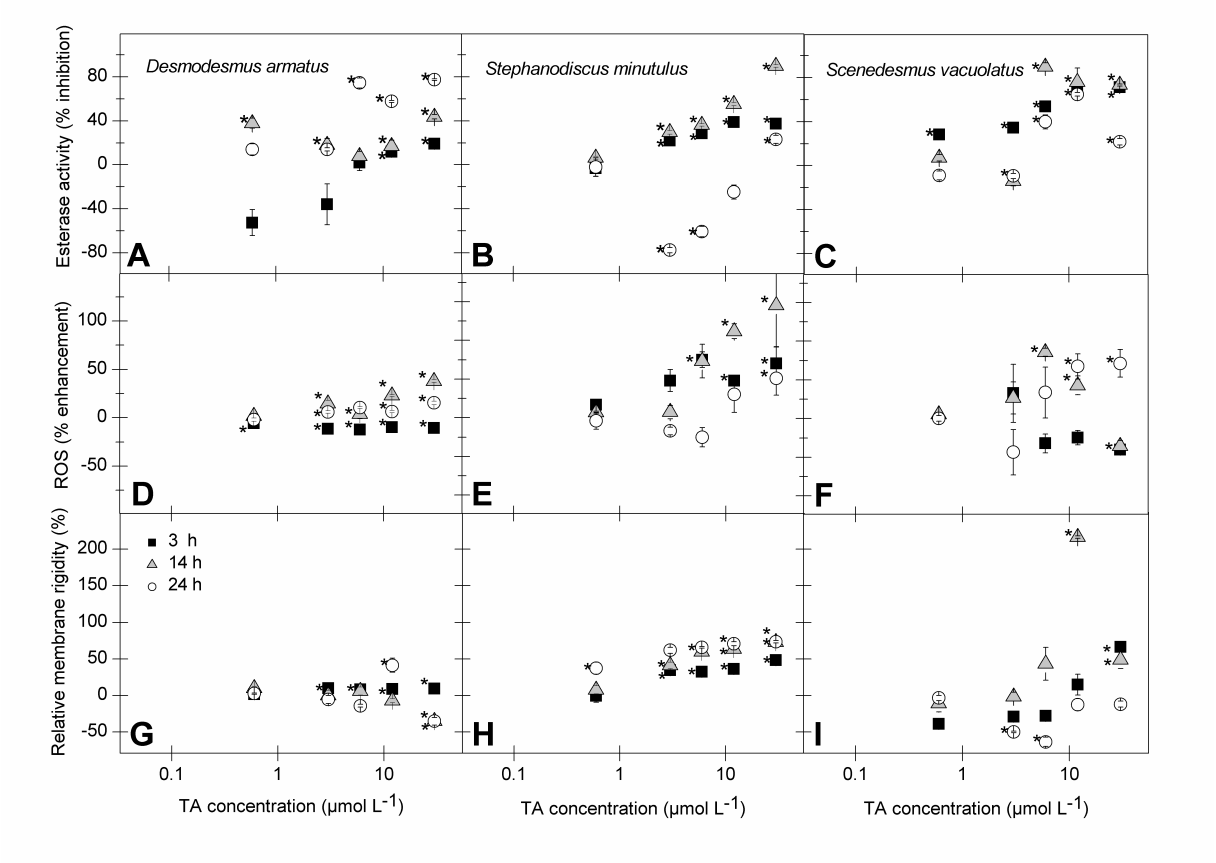

Figure 3:

Inhibition of esterase activity (A-C), production of ROS (D-F) and changes in membrane integrity (G-I) for the respective algal species given as % after exposure to a concentration series of TA (µmol L^{-1}) calculated from the means of the controls. Squares = 3 h exposure, triangles = 14 h exposure, circles = 24 h exposure. Asterisks indicate a significant difference ($p \leq 0.05$) compared to the controls. Error bars indicate the standard error (SE).

S. vacuolatus did not show a concentration-dependent response curve after a 14 h exposure (Fig. 3C), but revealed a 90% inhibition at 6 µmol L^{-1}. Therefore, we could not calculate EC_{20} and EC_{50} values from the 14 h treatment (Table 2).

Table 2: Variables and EC values for modelled concentration-response curves for the respective algal species, rounded to full numbers, except for α and EC_{20} (rounded to 0.5): All data are based on the equation: $Y= A_2 + (A_1-A_2)/(1 + (X/X_0)^{\alpha})$. A_1 and A_2 = % inhibition, X_0, EC_{20} and EC_{50} = µmol TA L^{-1}, R^2 = regression coefficient, p = significance of regression.

[a] = control data were excluded from the estimation. Absolute A_1 value was added to Y values for fitting the curve and subsequently subtracted again. [b] = control and 0.6 µmol L^{-1} data were excluded from the estimation. Absolute A_1 value was added to Y values for fitting the curve and subsequently subtracted again.

- : Fit not possible

*: Not conducted

Algal species	*Desmodesmus armatus*			*Stephanodiscus minutulus*			*Scenedesmus vacuolatus*		
Exposure Time (h)	3	14	24	3	14	24	3	14	24
Cell division									
A_1	*	*	0	*	*	0	*	*	0
A_2	*	*	39	*	*	18	*	*	100
α	*	*	1.5	*	*	1	*	*	2
X_0	*	*	2	*	*	1	*	*	18
EC_{50}	*	*	-	*	*	-	*	*	18
EC_{20}	*	*	2	*	*	-	*	*	9
R^2	*	*	0.95	*	*	0.36	*	*	0.96
p	*	*	<0.001	*	*	<0.001	*	*	<0.001
Photosynthetic activity									
A_1	-	-	-	0	0	0	-	0	0
A_2	-	-	-	12	69	27	-	100	100
α	-	-	-	2	0.5	1	-	2.5	2
X_0	-	-	-	17	267	4	-	17	19
EC_{50}	-	-	-	-	1419	-	-	17	19
EC_{20}	-	-	-	-	57	13	-	9.5	9.5
R^2	-	-	-	0.85	0.92	0.94	-	0.95	0.96
p	-	-	-	<0.001	<0.001	<0.001	-	<0.001	<0.001
Esterase activity									
A_1	-83 [a]	-	0	-2	0	-82 [b]	0	-	-6
A_2	29	-	100	38	100	39	100	-	68
α	1	-	0.5	2	1	2	0.5	-	1
X_0	2	-	3	3	8	13	5	-	8
EC_{50}	-	-	3	-	8	-	5	-	25
EC_{20}	29	-	0.5	3	2.5	27	0.5	-	12
R^2	0.73	-	0.82	0.85	0.97	0.98	0.95	-	0.38
p	<0.001	-	<0.001	<0.001	<0.001	<0.001	<0.001	-	0.003

Production of ROS

In all examined algal species, a significant level of ROS production was detected at higher concentrations of the allelochemical TA, but not for all exposure times (Fig. 3D-F). Absolute fluorescence values of the controls and inactivated controls are given as supplementary material (Table S1, subsequent to this chapter). ROS levels in *D. armatus* decreased slightly after a 3 h exposure (ca. 10%, Fig. 3D), and increased significantly (15 - 40%) with TA concentration after 14 and 24 h of exposure (Fig. 3D). In *S. minutulus* ROS increased significantly for all exposure times at 30 µmol L^{-1} TA (Fig. 3E). The production of ROS in *S. vacuolatus* showed neither a consistent pattern for concentration nor for exposure times. ROS levels significantly increased (up to 55%) only after a 24 h exposure at concentrations greater than or equal to 12 µmol L^{-1} TA (Fig. 3F).

Disturbance of membrane integrity

Exposure to TA did not result in a consistent effect on membrane integrity (Fig. 3G-I). Fluorescence values of the controls and inactivated controls can be seen in Table S1. Membrane rigidity in *D. armatus* cells increased significantly at TA concentrations greater than or equal to 3 µmol L^{-1} after 3 h, whereas after 14 h of exposure the membrane was significantly (up to 40%) more permeable at 30 µmol L^{-1} TA (Fig. 3G). After a 24 h exposure, significant increases and decreases in membrane rigidity were observed (Fig. 3G). In *S. minutulus* cells, membrane rigidity was positively correlated with TA concentration (Spearman`s rho correlation coefficient for 3 h exposure: 0.875, 14 h exposure: 0.97, 24 h exposure: 0.946), and rigidity increased with increasing exposure time (Fig. 3H). In *S. vacuolatus* cells, membrane rigidity was significantly increased at TA concentrations greater than or equal to 12 µmol L^{-1} TA after a 14 h exposure (Fig. 3I), whereas in the 24 h treatment, the membrane became more permeable with a significant peak (ca. 60%) at 6 µmol L^{-1} TA.

Inhibition of growth rate and photosynthetic yield

A significant inhibition of growth rates µ (d^{-1}) occurred in all three species (Fig. 4A-C). Inhibition was strongest in *S. vacuolatus* and weakest in *S. minutulus* (Fig. 4A-C). Modelled EC_{20} and EC_{50} values are given in Table 2. Growth rates µ (d^{-1}) of controls were 0.54 ± 0.04, 0.35 ± 0.1 and 2.44 ± 0.07 for *D. armatus*, *S. minutulus* and *S. vacuolatus*, respectively (data not shown). A significant inhibition of the photosynthetic yield was detected only for *S. minutulus* and *S. vacuolatus* (Fig. 4D-F). *D. armatus* displayed a weak but significant increase in its photosynthetic yield (Fig. 4D).

The inhibition of the photosynthetic yield of *S. minutulus* increased with exposure time. After 14 and 24 h exposures, inhibition became significant at TA concentrations greater than or equal to 3 µmol L^{-1} (Fig. 4E). *S. vacuolatus* was significantly inhibited (ca. 10%) after a 3 h exposure to only 30 µmol L^{-1} TA, whereby after 14 and 24 h exposures the photosynthetic yield was significantly inhibited by ca. 80 and 70% at 30 µmol L^{-1} TA (Fig. 4F). *S. vacuolatus* was the most sensitive species in terms of the inhibition of its photosynthetic yield, with EC_{20} values of 9.5 µmol L^{-1} in both, and EC_{50} values of 17 and 19 µmol L^{-1} after 14 and 24 h exposure periods (Table 2).

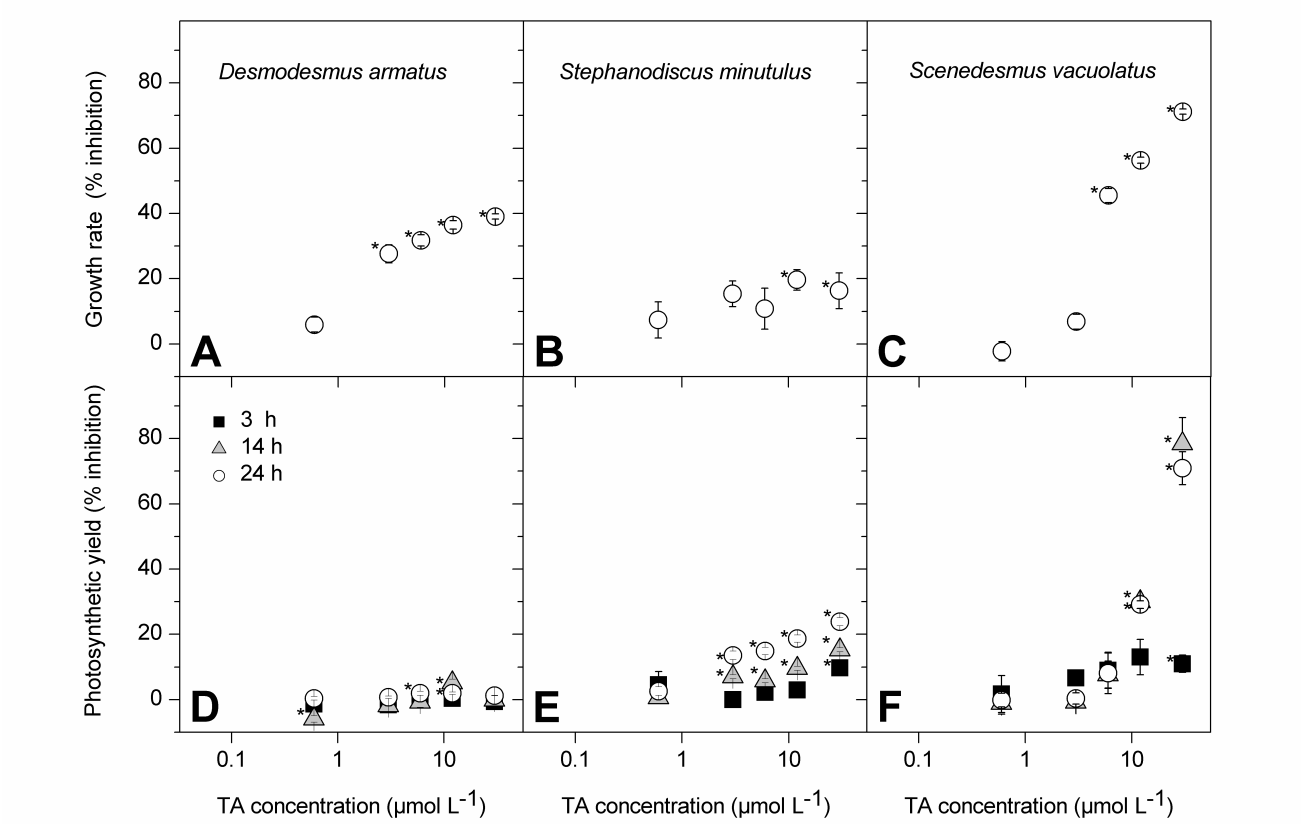

Figure 4:
Inhibition of growth rate (A-C) and photosynthetic yield (D-F) in % for the respective algal species after exposure to a concentration series of TA (µmol L^{-1}), calculated from the mean control. Squares = 3 h exposure, triangles = 14 h exposure, circles = 24 h exposure. Asterisks indicate a significant difference ($p \leq 0.05$) compared to the controls. Error bars indicate the standard error (SE).

Modelled baseline toxicity of TA

ChemProp revealed log K_{ow} values in the range of 1.8 up to 7.9 for TA, depending on the dissociation of this polyphenolic substance. These values correspond to EC_{50} values of 0.02 and 3505 µmol L^{-1}, respectively (after Altenburger et al., 2004), indicating toxicities in a range of several orders of magnitude.

Discussion

We identified changes in esterase activity and production of ROS as new responses of phytoplankton to polyphenolic allelochemicals by flow cytometry. Enzyme activity was inhibited in all three examined algal species at the highest tested TA concentrations after short time exposures (3 h). The production of ROS after TA exposures was also shown, but was less pronounced than the enzymatic response, and was only detectable within short (3 h) exposure periods for *S. minutulus*. TA showed no consistent effect on membrane integrity. In most cases, esterase activity was more sensitive and responded more rapidly than photosynthetic yield and growth rate (see Fig. 3 and 4). The inhibition of esterase activity thus proved to be a relatively rapid and likely primary response of algae to polyphenolic allelochemicals, and may therefore be an appropriate diagnostic tool for allelopathic effects in coexistence experiments.

Inhibition of esterase activity

Esterase enzymes exist in all organisms. They cleave ester-bondages into alcohols and acids and thereby fulfil a broad scope of duties. Some primary functions include the extraction of phosphate groups from molecules (alkaline phosphatase), the breakdown of fats and lipids (lipase) and detoxification (Tang et al., 2008). For cyanobacteria, esterase activity is a useful variable for the identification of metabolic activity (Brookes et al., 2000a) and can be measured by flow cytometry using the fluorescence dye FDA. The esterase-attributed conversion of FDA into fluorescein is correlated with photosynthetic rates (Brookes et al., 2000a) and nutrient-limited growth (Brookes et al., 2000b), with both processes known to be affected by polyphenolic allelochemicals (Körner and Nicklisch, 2002). Polyphenols are further known to act as potential enzyme inhibitors due to their complexation with polymers (Gross et al., 1996; He et al., 2006). Thus, the inhibition of esterase activity was a likely target of polyphenolic allelochemicals in algae, but was not evidenced in earlier studies.

We detected the inhibition of esterase activity by flow cytometry with the fluorescence dye FDA. FDA is also a marker for membrane integrity, because the FDA molecule and its cleavage product fluorescein cannot be contained by disturbed membranes and thus diffuses out of a cell (Persidsky and Baillie, 1977). However, we also used PI as a marker for membrane integrity, which was not correlated to esterase inhibition (data not shown). We can thus distinguish between effects on the enzyme and the membrane, concluding that the esterase activity was inhibited.

In four experiments (*D. armatus* 3 h treatment, *S. minutulus*, 24 h treatment; *S. vacuolatus* 14 and 24 h treatment; Fig. 3A-C) we observed an enhanced esterase activity at low TA

concentrations. Low concentrations of inhibitory chemicals can result in a positive stress response in plants and algae (e.g., Calabrese and Baldwin, 1998), a phenomenon known as hormesis (Paracelsus). However, considering increases of up to 80% (Fig. 3), a simple positive stress response is unlikely to be the only explanation. Enhanced esterase activities could also occur due to detoxification processes of esterases at low levels of toxic substances (Hong et al., 2008; Tang et al., 2008). Esterase activity also increased in the cyanobacterium *Microcystis aeruginosa* when exposed to the allelochemical ethyl 2-methyl acetoacetate (EMA, not a polyphenol). However, in this case, an increase was only observed after 24 h (a slight decrease occurred after 2 h) which was regarded as a defence mechanism against the stressor (Hong et al., 2008). As FDA is a marker for general esterase activity, and thus does not solely apply to detoxification enzymes, we cannot conclusively explain the increased esterase activity. In our study, there was no consistent pattern of increasing esterase activity with respect to exposure times. *D. armatus* revealed an enhanced esterase activity already after 3 h, whereas the two other species only showed increased esterase activities after 14 or even 24 h. These data imply that a fast and strong increase of esterase activity was correlated with a low sensitivity of the investigated algae in terms of growth and photosynthetic yield inhibition. This would corroborate the conclusion that increased esterase activity is used to activate the defence system against an allelochemical (Hong et al., 2008). However, since our results are not consistent for all three species, general conclusions may not be drawn. Further investigations with additional algal species are required in order to conclude whether enhanced esterase activity can be considered a general response to low concentrations of polyphenolic allelochemicals. Overall, these deviating patterns of increased esterase activity do not challenge the clearly demonstrated inhibition of esterase activity at high TA concentrations.

By calculating inhibition for naturally occurring concentrations of polyphenolic allelochemicals (3.6 µmol L^{-1} sensu Gross et al., 1996), we observed an inhibition of up to 51% of the esterase activity (Table 3). This inhibition might lead to a significant disadvantage for algal cells exposed to allelochemicals, since esterases fulfil a broad range of duties in algal physiology (Regel et al., 2002) and metabolic activity (Brookes et al., 2000a). In addition, we added TA only once during our experiments. This is in contrast to natural conditions where a continuous release of allelochemicals from macrophytes takes place. Nakai et al. (1999) found an inhibition of phytoplankton cultures only with the continuous release of allelochemicals, and thus single addition experiments might underestimate chronic effects (Ahlers et al., 2006). Accordingly, an inhibition of esterase activity in phytoplankton cells in dense, polyphenol-releasing macrophyte stands seems very likely.

Table 3: Estimated inhibition of esterase activity for naturally occurring concentrations of polyphenolic allelochemicals (3.6 µmol L^{-1} sensu Gross et al., 1996) for the respective algal species. Estimations are calculated with modelled fits of concentration-response curves (Table 2) after 3, 14 and 24 h exposure (Equation: $Y = A_2 + (A_1-A_2)/(1+(X/X_0)^{\alpha})$. - : fit not possible

Algal species	*Desmodesmus armatus*	*Stephanodiscus minutulus*	*Scenedesmus vacuolatus*
% inhibition (3 h)	-11	22	46
% inhibition (14 h)	-	31	-
% inhibition (24 h)	51	-73	17

Detection of ROS

The production of ROS in algal cells as a rapid response to stressors is highly probable (Szivak et al., 2009), and has been shown before in cyanobacteria, induced by the allelochemical EMA. ROS production should thus be one of the first physiological responses in algae exposed to polyphenolic allelochemicals (Szivak et al., 2009). However, in our study, only *S. minutulus* showed increased ROS levels after short time exposure (3 h) to TA (Fig. 3D-F). Based on the earlier response in terms of enzyme inhibition, ROS production may be a secondary effect, induced by the inhibition of detoxification enzymes such as esterases by polyphenolic allelochemicals.

Disturbance of membrane integrity

We could not detect a consistent pattern for the disturbance of membrane integrity. Considering the potential of TA to build complexes with proteins (Beart et al., 1985; Haslam, 1988; Smith et al., 2005), a disturbance of membrane integrity seems likely, taking into account the high quantity of membrane-bound proteins. Furthermore, TA is known to bind to lipid bilayers such as membranes (Simon et al., 1994). However, the only consistent concentration-dependent response in our study was detected in *S. minutulus* (Fig. 3H). This result partly confirms the *in vitro* study by Simon et al. (1994) where TA aggregated with lipid bilayers at lower concentrations (≤ 10 µM), but the bilayers became unstable and broke at higher concentrations

(≥ 50 µM). Our experiments may therefore have been maintained below a critical TA concentration. Studies on bacteria showed that polyphenols can reduce the membrane fluidity (i.e. the membrane becomes more rigid) due to the insertion or interaction in the outer polar zone of lipid bilayers (e.g., Tsuchiya, 1999). However, other studies have found increased membrane fluidity (i.e. the membrane becomes more permeable) in bacteria (due to polyphenols) and algae (due to EMA). The higher fluidity was explained by an increase of unsaturated fatty acids (Li and Hu, 2005; Smith et al., 2005; Yi et al., 2010). As the inactivated control cells in our study showed clear disturbances of membrane integrity, our test system appears to be valid. Thus, our results appear to be reliable and not methodological artefacts. We cannot, however, explain why *D. armatus* and *S. vacuolatus* membranes react differently at various TA concentrations and exposure times. Specificity of polyphenols to proteins could explain the inconsistent results (e.g., Beart et al., 1985; Haslam, 1988). Inconsistent concentration-response curves are generally common patterns in studies of allelopathy (Reigosa et al. 1999). However, the discrepancy between *in vitro* and *in vivo* studies was emphasized (Haslam, 1996), and no certain mode of action for polyphenols on membranes was found *in vivo*. This is likely due to the manifold mechanisms of interactions between polyphenols and polymers (hydrogen bonding, hydrophobic association, covalent bindings) which change with inherent parameters (e.g., substrate availability, chemical parameters) (Haslam, 1988; 1996). These parameters are difficult to control under *in vivo* conditions. Additional and more specific measurements (e.g., electron microscopy, nuclear magnetic resonance, X-ray) would be required to elucidate whether general modes of polyphenols on algal membranes exist.

Growth rate and photosynthetic yield

Growth rate inhibition reacted less sensitively than esterase activity, as the division of algal cells is a highly integrated factor based on many different physiological processes.

The PAM fluorescence measurements of the photosynthetic yield confirmed previous observations of highly species-specific inhibition of photosynthesis by allelochemicals (Körner and Nicklisch, 2002). For *S. minutulus* and *S. vacuolatus*, inhibition occurred after 3 h only at the highest concentration, whereas for *D. armatus*, photosynthetic yield remained uninhibited for the first 24 h (Fig. 4D-F). Interestingly, *D. armatus* simultaneously revealed a strong inhibition of its growth rate, indicating that the PS II may not be a primary target of TA, and may remain active in compromised cells. Inhibition of the photosynthetic yield may therefore be unsuitable as a general observation variable within the first 24 h of exposure to TA.

Modelled toxicity values

The modelled log K_{ow} values for TA resulted in EC_{50} values that reflect a range of toxicities over 5 orders of magnitude. These results illustrate that established models are insufficient in predicting the toxicity of complex, natural products. The complexity of polyphenols (Gross, 2003; Bauer et al., 2009), their possible rapid degradation (Bauer et al., 2012) and the large occurrence of hydroxyl groups (shifts of the Log K_{ow} value according to the pH) make it difficult to establish suitable models. Existing models suffer from the scarce availability of datasets for complex molecules. To obtain reasonable toxicity estimations, additional complex molecules must be incorporated into the existing databases. In conclusion, available models are not able to reflect complex natural compounds such as polyphenols, and thus a comparison of the results from this study to minimum toxicity values would not be reasonable.

Different sensitivities of the tested species

Our study confirmed Hilt et al. (2012), as sensitivities to TA were highly species-specific without showing consistent patterns between observation variables (Fig. 3 and 4). This holds true not only for each variable, but also for different exposure times. Inhibition of esterase activity was most pronounced in *S. vacuolatus* after a 3 h exposure (Fig. 3A-C), whereas *S. minutulus* and *D. armatus* were the most sensitive species following 14 h and 24 h exposures (respectively). We cannot exclude the possibility that different light intensities used for culturing (*S. vacuolatus* 370 µmol photons m^{-2} s^{-1}, *D. armatus* and *S. minutulus* each 80 µmol photons m^{-2} s^{-1}) influenced the cleavage of TA molecules. However, cleavage products of TA also inhibit photosynthetic yields and phytoplankton growth rates (Bauer et al., 2012). We thus consider our test conditions to be comparable to each other. Species sensitivities depend upon many factors which may occur simultaneously (Reigosa et al., 1996), and illustrate the complexity of toxicity of the natural chemical TA. However, in most cases (considering differing variables, TA concentrations and exposure times) *S. vacuolatus* and *D. armatus* were, respectively, the most and least sensitive species (Fig. 3 and 4). Still, general conclusions concerning the sensitivities of algal species towards allelochemicals may not be drawn, and conclusions therefore require specific variables, stressor amplitudes and exposure times. Furthermore, species-specific sensitivities are different in mixed cultures than in single-species experiments (Chang et al., 2012).

Suitability for coexistence experiments

In coexistence experiments, algae (single-species or mixed cultures) are placed in dialysis bags inside and outside macrophyte stands in lakes or in aquaria, with and without macrophytes, in order to detect the allelopathic effects of macrophytes on phytoplankton under *in situ*-like conditions. The assessment of esterase activity appears to be a novel and suitable approach which avoids interference by nutrient competition between macrophytes and phytoplankton; one of the main complications in coexistence experiments (Hilt and Gross, 2008).

The use of flow cytometry proved to be a highly promising diagnostic tool for studying the response of phytoplankton to allelochemicals. Future studies should apply our findings to coexistence experiments with single- and mixed phytoplankton cultures. Additional fluorescence markers for other physiological processes that might be targets of allelochemicals will be established in the near future, and may further contribute to a better understanding of the ecological relevance of allelopathy in aquatic environments.

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Table S1: Corrected absolute fluorescence values of the flow cytometric measurements for the controls and inactivated controls ± standard deviations. The outcomes of Mann-Whitney U tests between the controls and inactivated controls are indicated by p values. Controls of one marker at different exposure times do not underlie the same experiment and can therefor not be compared over time. For mode and indication of the markers see Table 1.

Algal species		*Desmodesmus armatus*			*Stephanodiscus minutulus*			*Scenedesmus vacuolatus*		
Marker	Exposure time (h)	Control	Inactivated control	p	Control	Inactivated control	p	Control	Inactivated control	p
FDA	3	159±50	-10±1	0.034	4846±359	359±148	0.05	3348±477	43±13	0.009
	14	3969±373	190±13	0.021	1391±50	33±3	0.046	6156±748	302±22	0.05
	24	680±103	-2±22	0.014	1299±206	417±68	0.05	601±106	11±1	0.05
HE	3	13±0.2	391±64	0.024	22±4.1	84±18	0.05	25±6	193±151	0.083
	14	19±1	1091±80	0.009	21±5.3	94±15	0.05	10±0.4	377±101	0.076
	24	10±0.2	283±87	0.014	24±2	105±9	0.05	21±0.3	1697±140	0.083
PI	3	12±0.3	550±41	0.021	17±0.5	118±70	0.05	8±2.5	1118±108	0.009
	14	15±2.6	898±141	0.021	37±4.8	66±15	0.05	13±1	367±93	0.05
	24	11±1.5	680±30	0.034	33±8.6	209±7.7	0.05	7±0.4	635±43	0.009

Chapter III:

Bacterial community composition associated with freshwater algae: species specificity versus dependency on environmental conditions and source community

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Abstract

We studied bacterial associations with the green alga *Desmodesmus armatus* and the diatom *Stephanodiscus minutulus* under changing environmental conditions and bacterial source communities, to evaluate whether bacteria-algae associations are species-specific or more generalized, and determined by external factors. Axenic and xenic algae were incubated *in situ* with and without allelopathically active macrophytes, and in the laboratory with sterile and non-sterile lake water and an allelochemical, tannic acid (TA). Bacterial community composition (BCC) of algae-associated bacteria was analysed by denaturing gradient gel electrophoresis (DGGE), nonmetric multidimensional scaling (NMDS), cluster analyses, and sequencing of DGGE bands. BCC of xenic algal cultures of both species were not significantly affected by changes in their environment or bacterial source community, except in the case of TA additions. Species-specific interactions therefore appear to overrule the effects of environmental conditions and source communities. The BCC of xenic and axenic *D. armatus* cultures subjected to *in situ* bacterial colonization, however, had lower similarities (ca. 55%), indicating that bacterial pre-colonization is a strong factor for bacteria-algae associations irrespective of environmental conditions and source community. Our findings emphasize the ecological importance of species-specific bacteria-algae associations with important repercussions for other processes, such as the remineralisation of nutrients, and organic matter dynamics.

Introduction

The phycosphere (the surface of an algal cell and its immediate environs; Bell and Mitchell, 1972) is a bacterial habitat distinct from the ambient water. In particular, the bacterial colonization of the phycosphere is closely related to and mediated by algal extracellular products and chemical signals (Bell et al., 1974). The influence of algae on bacteria can be positive (e.g., as a carbon source; Bell and Mitchell, 1972) or negative (e.g., when competing for nutrients; Bell et al., 1974). Associations between algae and bacteria therefore display the full possible spectrum of interactions, including symbiosis, parasitism, competition, commensalism and mutualism (Cole, 1982; Grossart, 1999; Sapp et al., 2007a). These interactions result in a strong linkage between heterotrophic bacteria and algae (Bell and Mitchell, 1972). Today, however, it remains unclear whether bacterial colonization of algal cells is species-specific (e.g., Grossart et al., 2005; Jasti et al., 2005; Rooney-Varga et al., 2005) or determined by environmental factors and bacterial source communities (e.g., Kaczmarska et al., 2005; Sapp et al., 2007b).

Jasti et al. (2005) isolated *Alexandrium* strains from the Gulf of Maine that shared many bacterial species with other *Alexandrium* cultures, regardless of the geographic origin. Bacteria associated with *Alexandrium* were different from other non-toxic phytoplankton species even when isolated from the same habitat (in this case the Gulf of Maine). Additionally, the species-specific bacterial colonization of marine diatoms (Schäfer et al., 2002; Grossart et al., 2005) and other algae (Hold et al., 2001) has been observed. On the contrary, two cultures of *Pseudonitzschia multiseries* which originated from the same strain have exhibited different epibiotic bacteria when maintained under different conditions (Kaczmarska et al., 2005). Other diatom cultures have also displayed changes in BCC over time after isolation, indicating environmental conditions as drivers of BCC (Sapp et al., 2007b). Bacterial source communities had negligible effects on BCCs associated with diatoms (Schäfer et al., 2002), but were found to be an important factor for bacterial associations with cyanobacteria (Dziallas and Grossart, 2011), even overruling environmental factors for BCC developments (Langenheder et al., 2006). Under *in situ* conditions, species-specific algal products, algal growth phase, changes in environmental conditions and bacterial source community potentially influence BCC associated with algae, but the specific contribution of each of these factors remains unclear.

Bacteria-algae interactions have numerous ecologically relevant functions including nutrient remineralisation, vitamin supply and carbon cycling (e.g., Bell and Mitchell, 1972; Bell et al., 1974; Cole, 1982). A specific effect may be the modulation of algal sensitivity towards

growth-inhibiting substances (Levy et al., 2009) such as allelochemicals (Keating, 1978; Bauer et al., 2010). Since different algal groups and species displayed different sensitivities towards allelochemicals (Körner and Nicklisch, 2002; Hilt, 2006; Hilt and Gross, 2008), it has been suggested that differences in BCC on algae influence allelochemical sensitivity (Casamatta and Wickstrom, 2000; Mulderij et al., 2005). Bauer et al. (2010) provided the first experimental evidence to demonstrate that xenic cultures of the green alga *Desmodesmus armatus* and the diatom *Stephanodiscus minutulus* exhibited varying sensitivities towards the allelochemical tannic acid (TA) depending on the presence of associated bacteria. Allelochemicals may further influence alga-associated BCC by favouring allelochemical-resistant and/or -degrading bacterial communities, since allelochemicals are known to represent specific bactericides (Walenciak et al., 2002). Allelochemical degradation or algal protection from allelochemicals may be performed by specific bacteria associated with algae, and hence algal sensitivities towards allelochemicals might be decreased.

We therefore hypothesize that algae with a known contrasting sensitivity towards allelochemicals (i.e., the green alga *D. armatus* and the diatom *S. minutulus*) would develop a distinct BCC for each algal species, whether under laboratory or *in situ* conditions, and independent of differences in environmental conditions and/or bacterial source community. The hypothesis was tested by comparing BCCs of free and attached bacteria after *in situ* incubation of initially axenic and xenic algae inside and outside of allelopathically active macrophyte stands (using flasks with membranes enabling the free entrance of natural lake bacteria). Furthermore, algal cultures were incubated in the laboratory by using sterile and non-sterile water from the same lake, and adding the allelochemical tannic acid.

Materials and Methods

Test organisms and culture conditions

Unicellular cultures of the green alga *Desmodesmus armatus* Chodat (strain 276-4e, SAG University of Göttingen, Germany) and the diatom *Stephanodiscus minutulus* Kütz (Kleve et Möller; strain 49.91, SAG University of Göttingen, Germany) were grown in MIII medium (Nicklisch, 1992) with a pH of 7.9 ± 0.1, modified after Körner and Nicklisch (2002). Both cultures were grown at 20 ± 0.5 °C with a 12:12 h light (80 µmol photons m^{-2} s^{-1}): dark cycle, shaken gently at 60 r.p.m., and maintained in our lab for several years. The two chosen algal species are characterized by a contrasting sensitivity towards allelochemicals: *D. armatus* is moderately insensitive towards polyphenolic allelochemicals whereas *S. minutulus* growth is inhibited by them (Körner and Nicklisch, 2002).

Algal concentration and growth rate

Growth rates of algae were determined by chlorophyll (chl) fluorescence measured with a Phyto-PAM (pulse-amplitude-modulated) fluorometer (Walz, Effeltrich, Germany). Algae were dark adapted for 15 min and subsequently 2 mL of algal suspension were placed in a cuvette equipped with a magnetic bar and a stamp. Minimal fluorescence F_0 (Schreiber, 1986) was determined and used as a proxy for the chl *a* content of algal cultures (Lürling and Verschoor, 2003). As we did not convert data into real chl *a* values, we subsequently use the term chl F_0. Growth rate μ was calculated as:

$\mu\ (d^{-1}) = \ln (\text{chl } F_0\ (t_x) - \text{chl } F_0\ (t_0)) / t$

where t is time in days, chl F_0 (t_0) the chl F_0 value at day 0, and chl F_0 (t_x) the chl F_0 value at day x. This calculation is valid for exponentially growing cultures measured always at the same time of day (Körner and Nicklisch, 2002).

Incubation experiments

We performed three algal incubation experiments (exp.) with bacteria: two *in situ* (exp. 1 and 3) and one under laboratory conditions (exp. 2) (Table 4). Exp. 1 aimed at testing whether xenic laboratory cultures of *S. minutulus* and *D. armatus* show species-specific BCCs and whether these change species-specifically when exposed to an allelopathically active macrophyte stand in a lake. In exp. 2, lake water with and without bacteria was added to xenic algal cultures in the laboratory. Environmental conditions thereby changed less drastically, and the effect of lake water without bacteria on BCC could be tested. Additionally, TA (a commercially available polyphenolic allelochemical) was added in order to directly test the effects of allelochemicals on BCC. Exp. 3 aimed at testing whether the bacterial colonization of axenic algae in lakes is species-specific, and whether BCC differs when samples are exposed inside and outside of an allelopathically active macrophyte stand. Because axenisation was unsuccessful for *S. minutulus*, exp. 3 was solely performed with *D. armatus* (Table 4).

Incubation of algal cultures in a lake (exp. 1 and 3)

Xenic (exp. 1) and axenic (exp. 3) algal cultures (70 mL) with a starting concentration of chl F_0 = 10 μg L^{-1} were incubated in 250-mL Schott bottles. The bottles were sealed with two filters (50 mm diameter and 5 μm pore size, Sartorius, Göttingen, Germany) on top of each other and clamped with rubber rings inside of the lid.

Table 4: Experimental design of algal incubations with lake bacteria under changing environmental conditions (for specification see Table 5). Exp. = experiment, MA = macropyhte allelochemicals, TA = tannic acid

	Algae species	Environmental conditions	Source of bacteria
Exp. 1	*D. armatus* *S. minutulus*	*In situ* (lake) + MA	Laboratory pre-settlement, lake water + MA
Exp. 2	*D. armatus* *S. minutulus*	Laboratory, Laboratory + TA	Laboratory pre-settlement, lake water
Exp. 3	*D. armatus* (axenic)	*In situ* (lake) + MA, *In situ* (lake)	Lake water + and - MA

This set-up permitted algal colonization by bacteria, but prevented the transfer of algae and other planktonic organisms (tested in pre-experiments and confirmed by microscopic analysis). Bottles were fixed with ropes and an anchor ca. 30 cm below the water surface and incubated in Lake Krumme Laake for three days. In exp. 1, bottles were only incubated within macrophyte stands, whereas in exp. 3, bottles were incubated inside and outside of macrophyte stands (see below, Table 4). Each experiment was performed with 4 replicates, whereby three (and only two in exp. 3) were further processed for molecular analyses. Lake Krumme Laake (52.4177°N, 13.6887°E, Berlin, Germany) is a sickle-shaped, eutrophic, and slightly humic shallow lake (1 ha), with a dense, monophyletic submerged macrophyte stand of the allelopathically active whorled water-milfoil *Myriophyllum verticillatum* L. in one bay, and a macrophyte-free bay at the other end (Hilt et al., 2006; Bauer et al., 2009). *In situ* conditions in the lake during the experiments differed from those in the laboratory mainly in terms of pH, concentrations of nutrients and dissolved organic carbon, daily variability of temperature (measured from variability in air temperature), light quality (not measured) and quantity (Table 5). Differences between the macrophyte-dominated and the macrophyte-free side in Lake Krumme Laake were negligible in terms of the above mentioned parameters (Table 5). Consequently, the presence of macrophyte allelochemicals should be the most prominent difference. Their direct detection was impossible due to interference with humic substances entering the slightly dystrophic lake from a surrounding peat bog (Appel et al., 2001; Hilt et al., 2006).

Table 5: Abiotic parameters and nutrient concentrations of Lake Krumme Laake for the respective dates of the performed experiments (exp.) and for laboratory conditions. +M = Macrophyte stand, -M = Macrophyte-free control site. [a] = daily mean values ± diurnal variations, measured at Lake Müggelsee 3 km away from Lake Krumme Laake. [b] = daily mean values, measured at 75 cm depth at Lake Müggelsee. [c] = during light phase. - = measurement not conducted.

Date	24.08.2009 (Start exp. 1)	27.08.2009 (End exp. 1)	10.08.2009 (exp.2)	17.08.2010 (+M, exp. 3)	17.08.2010 (-M, exp. 3)	Laboratory
pH	7.2	7.3	7.1	7.5	7.4	7.9 ± 0.1
Oxygen concentration (mg L^{-1})	8.6	9.5	6.5	6.2	6	-
Conductivity (µS cm^{-1})	159	158	158	162	162	-
Water temperature (°C)	21.2	21.3	23.2	23	22.5	20 ± 0.5
Air temperature (°C)	19 ± 6.5[a]	21.6 ± 8[a]	21 ± 6.6[a]	22.1 ± 5.4[a]	22.1 ± 5.4[a]	20 ± 0.5
Soluble reactive phosphorus (µg L^{-1})	9	6	4	<3	<3	1600
Total phosphorus (µg L^{-1})	41	46	37	29	24	1600
NH_4^+-N (mg L^{-1})	0.03	0.03	0.04	0.08	0.05	-
NO_3^--N (mg L^{-1})	0.01	<0.01	0.01	<0.01	<0.01	31
Dissolved organic carbon (mg L^{-1})	16.4	17.2	-	-	-	2.4
Solar radiation (µmol photons m^{-2} s^{-1})	65[b]	61[b]	97[b]	63[b]	63[b]	80[c]

Incubation of algal cultures in the laboratory (exp. 2)

Xenic algal cultures (40 mL) were diluted to chl F_0 = 20 µg L^{-1} and mixed with 40 mL of sterile lake water, lake water containing the natural bacterial community, and lake water containing the natural bacterial community with TA. The lake water was sampled on the first day of the experiment. All treatments were subsequently filtered at a low pressure through 5 µm (Sartorius, Göttingen, Germany) and 3 µm (Whatman, Göttingen, Germany) pore size filters. Treatments containing sterile lake water were additionally filtered through 0.2 µm filters (Whatman, Göttingen, Germany). For TA treatments, 10 mL of a TA (filling code: 403955/1 64400, Fluka, Karlsruhe, Germany) stock solution (0.375 g L^{-1}) prepared in modified MIII medium (see above) were added to arrive at a final TA concentration of 24.5 µmol L^{-1}. The TA concentration was chosen based on pre-experiments with respect to non-lethal growth inhibition of the algae. These experiments lasted for five days, whereby experimental conditions were the same as culture conditions. Experiment 2 was performed with three replicates (two were used for DGGE).

Axenisation of cultures

Cultures of *D. armatus* were grown on agar plates with agar noble (Roth, Karlsruhe, Germany), and single colonies were transferred with toothpicks to a liquid-modified MIII medium under sterile conditions before treatments. For axenisation, 1.8 mL of algal culture were transferred into 2-mL Eppendorf tubes (Eppendorf, Hamburg, Germany), vortexed for 20 sec and subsequently placed in a Sonopuls UW 2070 ultrasonic bath (Bandelin, Berlin, Germany) for 30 sec in order to break up cell conglomerations. Afterwards, tubes were centrifuged (Thermoscientific, Fresco 17, Bremen, Germany) for 5 min at 6,000 x g, the supernatant was discarded and replaced with 1 mL of modified MIII medium. This step was repeated 3 times. At the 4th round, instead of medium, 1 mg mL^{-1} lysozym-solution (dissolved in the medium, Sigma Aldrich, Karlsruhe, Germany) was added, and tubes were incubated for 60 min at 37 °C. Next, tubes were vortexed several times with delays and the algal solution was transferred into 300-mL Erlenmeyer flasks containing 50 mL of modified MIII medium including 250 µg mL^{-1} ampicillin (Sigma-Aldrich, Karlsruhe, Germany), 50 µg mL^{-1} gentamycin (Roth, Karlsruhe, Germany), 100 µg mL^{-1} kanamycin (Sigma-Aldrich, Karlsruhe, Germany), 500 µg mL^{-1} neomycin (Roth, Karlsruhe, Germany), 100 µg mL^{-1} tetracycline (Sigma-Aldrich, Karlsruhe, Germany), 100 µg mL^{-1} streptomycin (Fluka, Karlsruhe, Germany) and 50 µg mL^{-1} chloramphenicol (Sigma-Aldrich, Karlsruhe, Germany). Antibiotic concentrations and mixtures were chosen based on pre-experiments. After three days, 1 mL of

the culture was removed and stained with DAPI (see below). In order to remove all antibiotics, cultures were sub-cultured two more times with medium before being used in the experiments. If bacteria appeared in the DAPI-stained samples, 1.8 mL of the culture were transferred into a new 2-mL Eppendorf tube and the whole procedure was repeated. When DAPI staining did not reveal any bacterial contamination, 100 µL of the suspended culture were removed and mixed with 0.1 µL of the more sensitive SYBR Green I solution (Sigma-Aldrich, Karlsruhe, Germany) in order to ensure the axenic status. After incubation for 10 min, the SYBR Green/algae mixture was filtered at a low pressure onto a black 0.2 µm pore-size Nucleopore filter (Whatman, Göttingen, Germany) and examined for bacterial contamination with a fluorescence microscope at 1000x magnification.

Bacterial abundance

One mL of the algal cultures was filtered at low-pressure with black 0.2 µm pore-size Nucleopore filters (Whatman, Göttingen, Germany) after staining for 15 min with 100 µL of the DAPI solution (Merck, Darmstadt, Germany). Filters were transferred to slides, and bacteria on 10 randomly chosen squares were counted at 1000x magnification with an Axioskop epifluorescence microscope (Zeiss, Göttingen, Germany).

DNA/RNA extraction

In order to determine which bacteria are active (and not only present), rRNA was transcribed into cDNA (which was applied in DGGE analyses) after DNA/RNA extraction. Samples of all treatments were subsequently low-pressure filtered on 5, 3, and 0.2 µm pore-size Nucleopore filters (Whatman, Göttingen, Germany) to obtain algae-associated (5 and 3 µm) and free-living (0.2 µm) bacteria. Filters were stored at -80 °C prior to DNA/RNA extraction. DNA/RNA was extracted following methods described by Allgaier and Grossart (2006a). Briefly, filters were mechanically disrupted for 10 min at 800 x g using 0.25 g of combusted Zirconium beads (Roth, Karlsruhe, Germany) and an extraction buffer containing 500 µL TENP-buffer, 500 µL phenol-chloroform-isoamylalcohole (PCI, 25:24:1; Sigma-Aldrich, Karlsruhe, Germany) and 60 µL 25% SDS solution (Merck, Darmstadt, Germany). Heating at 60 °C for 10 min was followed by centrifugation at 9,600 x g for 6 min at 4 °C. Following this, supernatants were washed several times with 500 µL PCI until no precipitate in the interphase could be observed. DNA/RNA mixtures were precipitated with 120 µL 3 M Na-acetate-solution and 1 mL ice-cold 96% ethanol (Merck, Darmstadt, Germany) at -20 °C for 2 h. Samples were then centrifuged at 17,000 x g for 90 min at 4 °C. The supernatant was

carefully discarded, and the pellet was washed with 850 µL of 80% ice-cold ethanol (Merck, Darmstadt, Germany) and incubated for 10 min on ice. Samples were centrifuged at 17,000 x g for 15 min at 4 °C, the supernatant was discarded and the pellet dried. Finally, the pellet was resolved for 2 h in 80 µL PCR-water (Sigma-Aldrich, Karlsruhe, Germany) and frozen at -80 °C until further treatment.

DNA digestion and cDNA synthesis

Twenty µL of the DNA/RNA extracts were subjected to DNA digestion using the Turbo-DNase kit (Ambion, Darmstadt, Germany) following the manufacturer's instructions. RNA purity was validated using universal primers (as described below) and remaining DNA contaminations were digested by repeating the DNase treatment. For cDNA synthesis, 5 µL of RNA were mixed with 1 µL primer 907r (20 pmol μL^{-1}, Muyzer et al., 1993) and 5.5 µL nuclease-free water, and incubated for 5 min at 70 °C. After chilling samples on ice, 4 µL dNTPs (2.5 mM for each dNTP), 2 µL 10x ArrayScript buffer, 1 µL RNase inhibitor and 1 µL reverse transcriptase (both ArrayScript, Darmstadt, Germany) were added, and incubated at 42 °C for 2 h. The reaction was inactivated by incubating samples for 5 min at 95 °C.

PCR reactions

To ensure successful DNA digestion and cDNA synthesis, and for reamplification of excised DGGE bands, PCR reactions were performed in a Thermocycler (JenaAnalytik, Jena, Germany). A total volume of 50 µL contained 5 µL 2.5 mM dNTPs, 2 µL 50 mM $MgCl_2$, 5 µL PCR buffer (Bioline, Luckenwalde, Germany), 0.5 µL Red Taq DNA-Polymerase (1 U μL^{-1}, Bioline, Luckenwalde, Germany) and 1 µL BSA (30 g L^{-1}). Approximately 20 ng DNA and 0.5 µL of each 20 pmol μL^{-1} bacteria-specific primer 341f (Muyzer et al., 1993) and 907r (Muyzer et al., 1995) were added to the reaction mix. After an initialising step at 95 °C for 3 min, 30 cycles of denaturising at 95 °C for 1 min, primer annealing at 55 °C for 1 min and elongation at 72 °C for 2 min were run. Finally, PCR products were elongated at 72 °C for 15 min. For PCR reactions after cDNA synthesis (DGGE preparation) the forward primer 341 with a 40 bp GC-rich nucleotide sequence (GC clamp) at the 5-end was added to stabilize the migration of DNA fragments in DGGE gels (Muyzer et al., 1993).

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE gels (7% v/v polyacrylamide) had a denaturing gradient of urea and formamide ranging from 40 to 70%. Approximately 500 ng of amplified DNA were used. After

electrophoresis at 100 V for 26 h, DGGE gels were stained with SYBR Gold solution (Invitrogen, Darmstadt, Germany) and photographed under UV-light with an AlphaImager TM 2200 apparatus (Proteinsimple, San Jose, USA). Prominent bands were excised with a scalpel from DGGE gels and resolved in 30 µL PCR water. Two µL of template were reamplified (see above). DNA was precipitated with PEG (Rosenthal et al., 1993), and sequenced with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems), following the manufacturer's instructions. The nearest relatives of obtained sequences were identified by BLAST searches (http://www.ncbi.nlm.nih.ov/blast), and deposited under accession numbers HE855859-HE855878 at the European Nucleotide Archive. Presence/absence matrices were performed with GELCOMPAR II, version 3.5 (Applied Maths); bands were marked and assigned to band classes manually, and normalized with the marker lanes (a mixture of 4 cultivated bacterial strains). Due to problems in combining different gels, all samples of *D. armatus* in all experiments were run on a single DGGE gel. For NMDS plotting, presence/absence tables were imported to the ordination software PC-ORD version 5.01 (MJM Software Design) and applied as described in Allgaier and Grossart (2006b) by calculating the Sørensen similarity index. The resulting 2D plots were graphically modified with the software Origin (Microcal, Northhampton, MA, USA). Similarity matrix dendrograms for attached and free bacteria fractions of all experiments for *D. armatus* were calculated by applying Dice correlation coefficients and UPMGAs with GELCOMPAR II, version 3.5 (Applied Maths).

Phylogenetic analysis of DGGE band sequences

The phylogenetic affiliation of sequenced DGGE bands was calculated using the ARB software package (Ludwig et al., 2004). Obtained sequences were aligned with an ARB SINA aligner, available at http://www.arb-silva.de. Sequences with several dubious bases were excluded from the tree. The resulting alignment was merged with an ARB SSU rRNA database (March 2012) on a local computer, manually adjusted, and added to the basic SSU rRNA, applying a maximum-parsimony quick-add function. A maximum-likelihood tree of identified proximate relatives with a minimum sequence length of 1200 nucleotides (nt) was calculated using an RAXML algorithm and the GTRCAT and GAMMAI model. To validate tree topology we used a bootstrapping analysis with 1000 replicates. Short DGGE sequences were added to the tree with the highest likelihood by applying a parsimony quick-add tool.

Statistical analyses

Algal growth rates μ (d^{-1}) of different treatments were compared by Mann-Whitney-U (MWU) tests, and differences in bacterial abundances were tested with t-tests. These statistical analyses were performed with the software package PASW 17 (SPSS).

Differences between banding patterns were tested by ANOSIM with the software PRIMER 6 version 6.1.12 (PRIMER-E), after calculating a presence/absence resembling matrix following Sørensen. The sample statistic (R) indicates the degree of separation between groups, where 1 stands for total separation, and R = 0 means no separation. Because ANOSIM compares dissimilarities between groups with dissimilarities within groups, samples that were not replicated (xenic samples before the exposure in exp. 1 and 2) could not be tested separately. Significance levels for all tests were set to $p \leq 0.05$.

Results

Bacterial colonization of xenic algae *in situ* (exp. 1)

A comparison of DGGE banding patterns by NMDS plotting before and after the exposure in Lake Krumme Laake revealed completely overlapping clusters (Fig. 5). Both algal species and the free and attached bacteria fraction, however, clustered separately (Fig. 5).

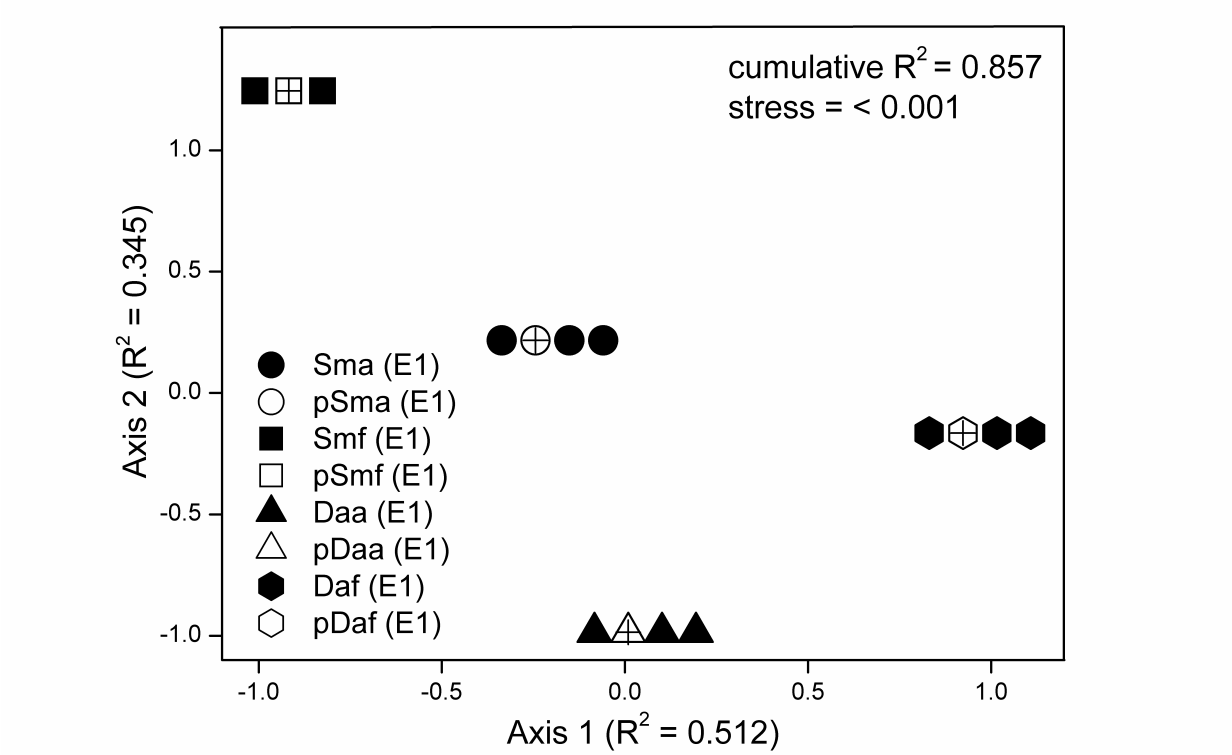


Figure 5:

NMDS ordinations based on the presence or absence of bacterial band classes in DGGE analysis of experiment 1 (exposure of xenic algal cultures in Lake Krumme Laake). Legend codes: p = before start of the experiment, Da = *Desmodesmus armatus,* Sm = *Stephanodiscus minutulus*, f = free bacterial fraction, a = attached bacterial fraction. A + indicates the centre of overlapping plots which are displayed next to it. The experiment number is indicated in the brackets.

By compiling groups of banding patterns by algal species and bacterial fraction, significant differences were observed between all groups tested (ANOSIM, Table 6). Bacterial abundance in exponentially growing *D. armatus* cultures ($\mu = 0.74 \pm 0.13$ d^{-1}) did not differ significantly before and after the incubation in the lake (t-test, $p = 0.436$, 115,550 ± 48,352 and 142,717 ± 77,354 cells mL^{-1}, respectively, data not shown). In parallel, bacterial abundances in *S. minutulus* cultures, which grew much more slowly ($\mu = 0.1 \pm 0.05$ d^{-1}), significantly increased after incubation in the lake (t-test, $p < 0.0001$, from 78,301 ± 53,520 to 481,397 ± 124,525 cells mL^{-1}, data not shown). Altogether, 16 DGGE phylotypes (band classes) were generated in exp. 1 (data not shown).

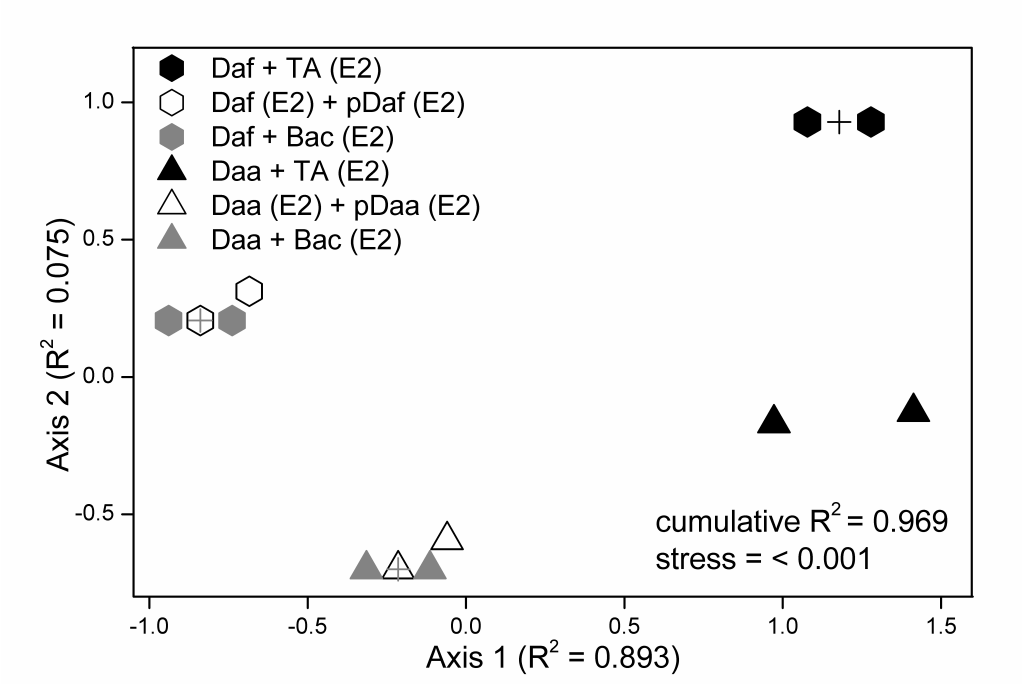
Bacterial colonization of xenic algae in the laboratory (exp. 2)

Bacterial fractions (free and attached) of both algae remained highly similar (> 80 and 90% similarity for *S. minutulus* and *D. armatus*, respectively, data not shown) after the addition of sterile lake water. These treatments were therefore combined in Fig. 6A and B.

Table 6: Results of ANOSIM calculations. The groups were created based on NMDS plotting results with the factors algal species, bacterial fraction, TA exposure, and number of experiment. All other variables did not show separate clusters in the NMDS plots. The first two letters in the column "Groups compared" refer to the algal species (Da = *Desmodesmus armatus*, Sm = *Stephanodiscus minutulus*); bacterial fractions are indicated by a = attached and f = free; + TA indicates exposure to tannic acid, and the experiment number is indicated in the brackets.

Groups compared	Sample statistic (R)	p
Sma (E1)/Smf (E1)	1	0.029
Sma (E1)/Daa (E1)	1	0.029
Sma (E1)/Daf (E1)	1	0.029
Smf (E1)/Daa (E1)	1	0.029
Smf (E1)/Daf (E1)	1	0.029
Daa (E1)/Daf (E1)	1	0.029
Sma + TA (E2)/Sma (E2)	1	0.048
Sma + TA (E2)/Smf + TA (E2)	0.25	0.667
Sma + TA (E2)/Smf (E2)	1	0.067
Sma (E2)/Smf + TA (E2)	0.818	0.048
Sma (E2)/Smf (E2)	1	0.008
Smf (E2) + TA/Smf (E2)	0.786	0.067
Daa (E2) + TA/Daf + TA (E2)	1	0.333
Daa (E2) + TA/Daf (E2)	1	0.048
Daa + TA (E2)/Daa (E2)	1	0.067
Daf + TA (E2)/Daf (E2)	1	0.048
Daf (E2) + TA/Daa (E2)	1	0.067
Daf (E2)/Daa (E2)	1	0.008
Daa (E1)/Daa (E2)	1	0.008
Daa (E1)/Daa (E3)	1	0.029
Daa (E1)/Daa + TA (E2)	1	0.067
Daa (E2)/Daa (E3)	1	0.008
Daa (E3)/Daa + TA (E2)	1	0.067
Daf (E3)/Daf (E2)	1	0.018
Daf (E3)/Daf (E1)	1	0.1
Daf (E3)/Daf + TA (E2)	1	0.1
Daf (E2)/Daf (E1)	1	0.018
Daf (E1)/Daf + TA (E2)	1	0.1

(A)



(B)

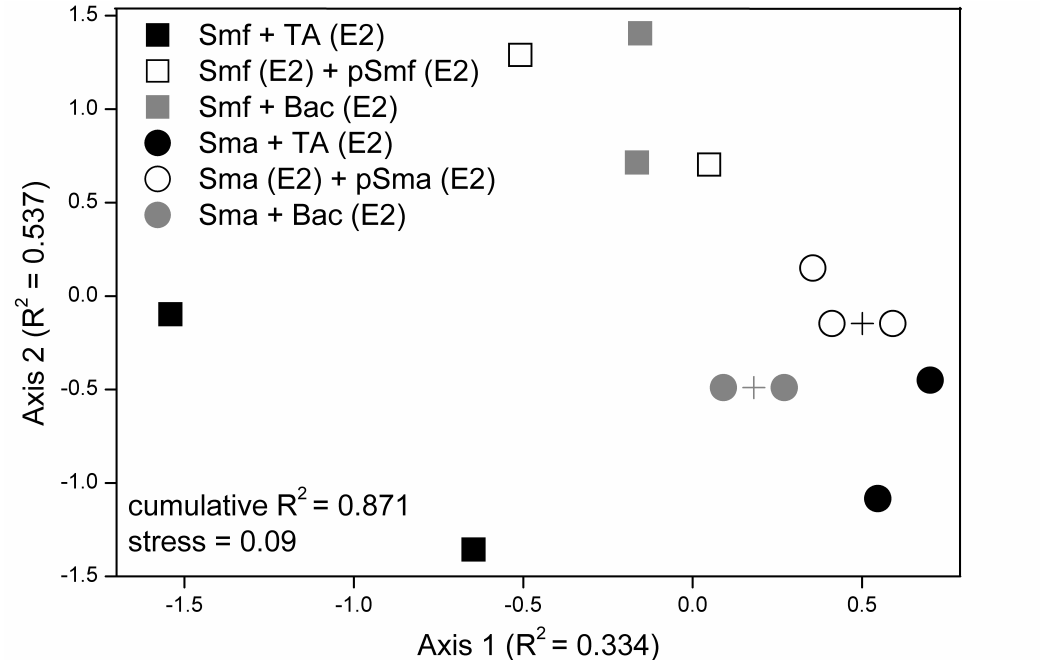


Figure 6:

NMDS ordinations of (A) = *Desmodesmus armatus* and (B) = *Stephanodiscus minutulus* based on the presence or absence of bacterial band classes in DGGE analysis of experiment 2 (exposure of xenic algal cultures with lake water in the laboratory). Legend codes: p = before start of the experiment, Da = *Desmodesmus armatus,* Sm = *Stephanodiscus minutulus*, f = free bacterial fraction, a = attached bacterial fraction, + Bac = inocula with bacteria, + TA = 24.5 µmol L^{-1} tannic acid. A + indicates the centre of overlapping plots which are displayed next to it. The experiment number is indicated in the brackets.

In *D. armatus*, no differences were found between the BCC of treatments with sterile and non-sterile lake water (Fig. 6A). The addition of TA, however, resulted in separate clusters for both bacterial fractions (Fig. 6A). In *S. minutulus,* small differences between treatments with sterile and non-sterile lake water appeared in the attached fraction, whereas in the free fraction replicates revealed differences in the same range as differences between these two treatments (Fig. 6B). However, TA addition resulted in separate clusters for both bacterial fractions, whereby the attached fraction showed a less distinct cluster after TA addition than the free fraction, whose replicates did not cluster close to each other (Fig. 6B). After combining proximate NMDS plots (BCC before treatments, treatments with sterile, and treatments with non-sterile lake water), most of the compared groups exhibited significant differences (ANOSIM, Table 6). The bacterial abundances of *D. armatus* cultures did not show significant differences at the end of the experiment between sterile and non-sterile lake water (t-test, $p = 0.528$, Fig. 7A). Bacterial abundance was more than three times higher in the TA treatment (t-tests, $p = 0.013$ and 0.01) than with sterile and non-sterile lake water, respectively (Fig. 7A). Growth rates of *D. armatus* were the same (ca. 0.55 d^{-1}) in sterile and non-sterile lake water (MWU test, $p = 0.376$), but decreased significantly in the TA treatment ($\mu = 0.1$ d^{-1}; Fig. 7C, MWU, $p = 0.05$ each, compared to sterile and non-sterile lake water).

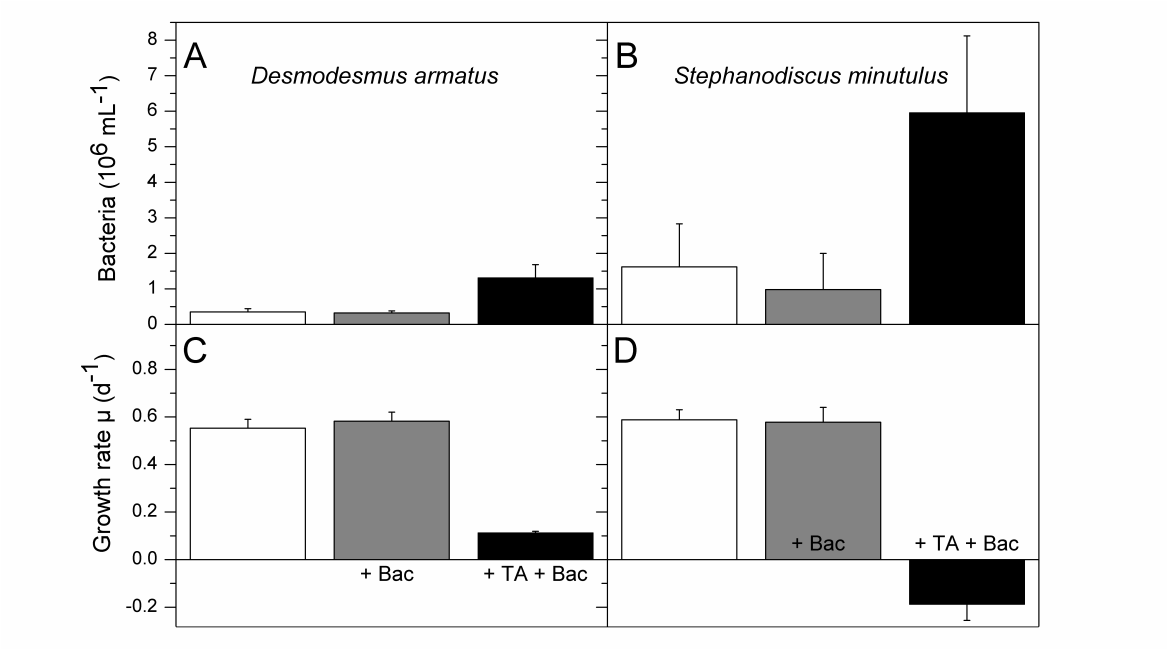


Figure 7:

Bacterial abundances and growth rates of *D. armatus* (A, C) and *S. minutulus* (B, D) cultures in experiment 2 (exposure of xenic algal cultures with lake water in the laboratory). Error bars indicate the standard deviation. Sample prefixes: Da = *Desmodesmus armatus,* Sm = *Stephanodiscus minutulus*, + Bac = inocula with bacteria, + TA = 24.5 µmol L^{-1} tannic acid.

Bacterial abundances of *S. minutulus* cultures were similar between treatments with sterile lake water and lake water with natural bacteria (t-test, $p = 0.338$), and significantly higher in the treatments with TA than in sterile and non-sterile lake water treatments (t-test, $p = 0.032$ and 0.036, respectively; Fig. 7B). Growth rates of *S. minutulus* were comparable for both treatments without TA addition (MWU, p = 0.827, Fig. 7D), but were negative with TA addition (MWU, p = 0.05 each, compared to sterile and non-sterile lake water; Fig. 7D). Overall, 22 phylotypes were generated by DGGE (data not shown).

Bacterial colonization of axenic *D. armatus* in the lake (exp. 3)

Free and attached bacterial fractions clustered separately (Fig. 8, ca. 50% similarity between them, data not shown). All attached BCC clustered in one single point, regardless of their exposure location (in the macrophyte stand vs. macrophyte-free bay, Fig. 8).

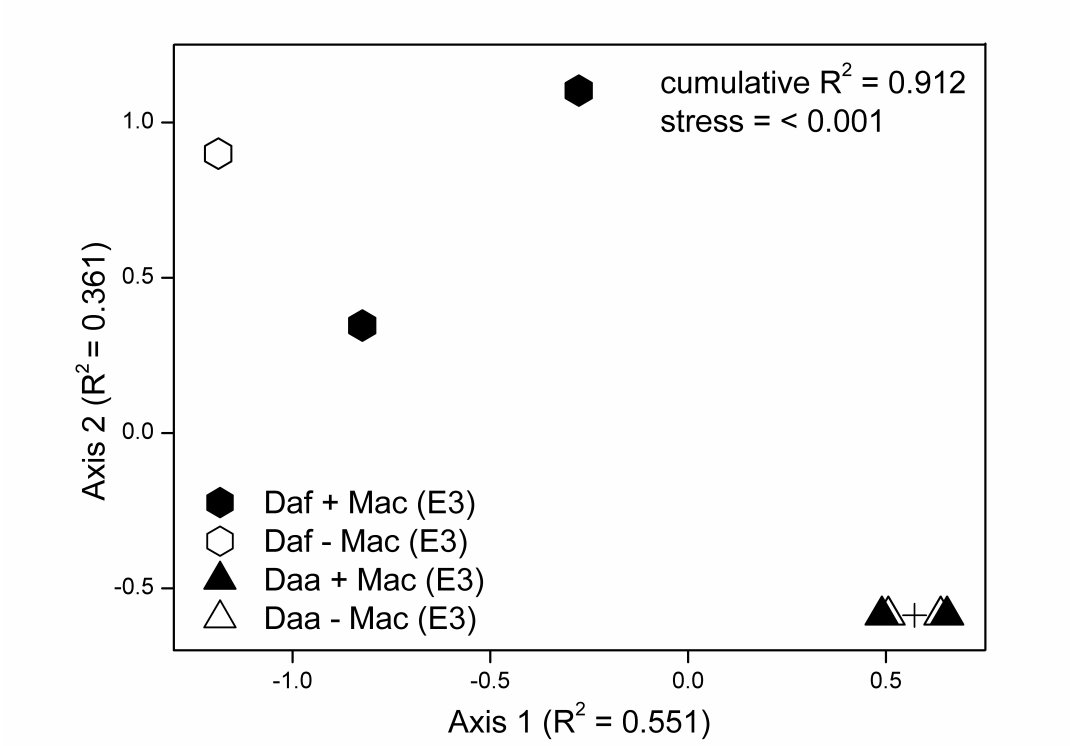


Figure 8:

Demosdesmus armatus: NMDS ordinations based on the presence or absence of bacterial band classes in DGGE analysis of experiment 3 (exposure of axenic *D. armatus* cultures in Lake Krumme Laake). Sample prefixes: Da = *Desmodesmus armatus*, f = free bacterial fraction, a = attached bacterial fraction, + Mac = exposed in the stand of *M. verticillatum*, - Mac = macrophyte-free control bay. A + indicates the centre of overlapping plots which are displayed next to it. The experiment number is indicated in the brackets.

The free bacterial fraction did not cluster together, but likewise no pattern of exposure location was found (Fig. 8). The natural bacterial communities in the controls of both sites did not show significant differences (ANOSIM, R = 0.5, p = 0.333, data not shown). The sample

"free 1 – Mac" smeared in the DGGE gel, and was therefore excluded from the analysis. *D. armatus* grew exponentially, with $\mu = 0.71 \pm 0.2$ d^{-1} in the macrophyte stand and $\mu = 0.8 \pm 0.003$ d^{-1} at the macrophyte-free control site (data not shown). Hence, no significant differences in algal growth could be detected between incubation sites (MWU, $p = 0.513$). Eleven phylotypes were generated by DGGE (data not shown).

Comparison of all experiments with the green alga *D. armatus*

Community composition of free bacteria in all *D. armatus* experiments showed at least 65% similarity (Fig. 9A), except for TA treatments in exp. 2 (ca. 40% similarity to all other samples). Samples of each experiment clustered together (except for the TA treatments). Free bacteria of *D. armatus* between exp. 1 and 3, and the TA treatment of exp. 2 compared to the free fraction of exp. 1 and 3 did not show significant differences, but exhibited an R score of 1 (Table 6). Community composition of attached bacteria in all *D. armatus* experiments revealed a similarity of ca. 45% (Fig. 9B), but the BCC of all non-TA treatments were more similar (ca. 55% similarity; Fig. 9B). All groups (see Table 6 for grouping) of attached bacteria were highly dissimilar by exhibiting an R score of 1 with p values between 0.008 and 0.067 (Table 6). For both free and attached bacteria, 17 phylotypes were generated by DGGE (data not shown).

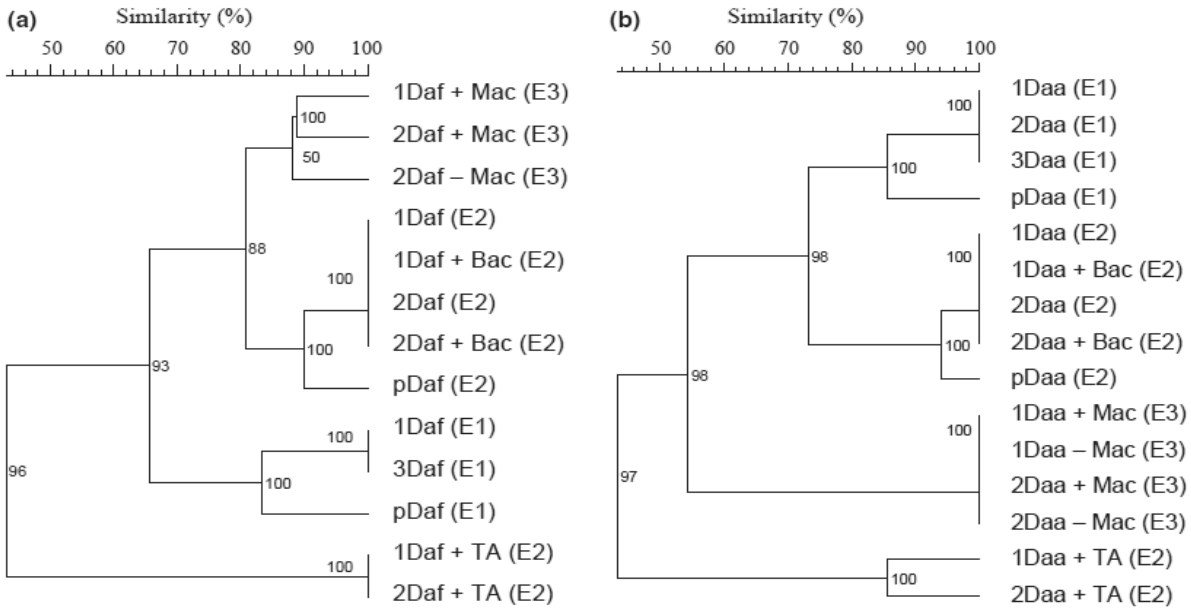


Figure 9:

Desmodesmus armatus: Cluster analysis of (a) free and (b) attached bacteria based on the presence or absence of bacterial band classes in all experiments (run on a single DGGE gel), expressed as % similarity. Sample prefixes: numbers = replicates, + Mac = stand of *M. verticillatum*, - Mac = macrophyte-free control bay, + Bac = inocula with bacteria, + TA = 24.5 µmol L^{-1} tannic acid. The experiment number is indicated in the brackets. Numbers in front of the branches describe cophenic correlations.

Phylogenetic affiliation of sequences

Figure 10 depicts a maximum-likelihood tree that summarizes the phylogenetic relationship of partial 16S rRNA genes DGGE bands obtained from the algal treatments and controls. The backbone tree was constructed of nearly full length 16S sequences (1200 nt). Twenty-six DGGE phylotypes were found in all *D. armatus* experiments (data not shown), of which nine could be sequenced (Fig. 10). Cultures of *S. minutulus* revealed 9 and 15 DGGE phylotypes for exp. 1 and 2, respectively, of which 8 from exp. 2 alone could be sequenced (Fig. 10). Three sequences were obtained from the controls (exp. 3, Fig. 10). Fourteen out of 20 sequences grouped within the closely related β- and γ-*Proteobacteria*. Relationships to *Burkholderiaceae*, *Comamonadaceae* and *Nitrosomonadaceae* were primarily classified within the β-*Proteobacteria*, while sequences within the γ-*Proteobacteria* were affiliated with *Rheinheimeria* sp. and *Acinetobacter* sp. Two sequences clustered within the α-*Proteobacteria,* with close relationships to *Sphingopyxis* sp. and *Sphingomonas* sp. Two further DGGE sequences were affiliated with *Spirosoma* sp. and *Flavobacterium* sp., belonging to the phylum *Bacteriodetes*. Finally, two DGGE bands were distributed among the ε-*Proteobacteria* and the *Deinoccoccales*. Interestingly, 12 out of 20 sequences clustered together with known polyphenol/humin degraders (Fig. 10).

Figure 10:

Maximum likelihood tree of partial and nearly full length 16S sequences obtained from algal treatments. Each sequence from this study is bold-faced. The number of the experiment (= exp) which is given in the name corresponds to the experiment described in the text. The tree topology was tested by bootstrapping analysis, and bootstrap values above 50% are shown. The scale bar represents 10 substitutions per 100 nucleotides. Da = sequence derived from *D. armatus*, Sm = sequence derived from *S. minutulus*, control = sequence derived from bottles filled with medium without algae. Rough classification of polyphenol/humin degraders based on BLAST searches: Grey highlighted = known humin/polyphenol degraders.

Discussion

Species-specific bacterial colonization of *D. armatus* and *S. minutulus*

Our study indicates a strong species-specific bacterial colonization of two tested freshwater algal species, the green alga *D. armatus* and the diatom *S. minutulus*. The results thus corroborate numerous studies which have indicated that algae may at least partly determine BCC (Bell and Mitchell, 1972; Riemann et al., 2000; Fandino et al., 2001; Grossart et al., 2005).

Firstly, minor changes in the BCC of both tested xenic algae, in conjunction with low similarities between BCC of both tested algal species after exposure in the lake (exp. 1) and with the addition of lake water (exp. 2), indicate a strong species-specific bacterial association. Furthermore, only two of the 21 bacterial phylotypes from controls (flasks without algae, exp. 1) were also found in the algal treatments, which again indicates a high host-specificity of alga-associated BCC (data not shown). Secondly, colonization of initially axenic *D. armatus* in a lake (exp. 3) shared at least 65 and 55% bacteria species present in the xenic laboratory cultures (free and attached fraction, respectively, Fig. 9A, B), strongly suggesting a species-specific association for numerous bacteria. These high similarities, consistent between all experiments and approaches, were surprising considering that our xenic algal cultures were maintained for some years in the laboratory under constant incubation conditions. Thus, one might expect that a laboratory-adapted BCC may have emerged as suggested in several earlier studies (Janse et al., 2000; Grossart et al., 2005; Jasti et al., 2005; Sapp et al., 2007a; b). It is clear that the effects of algal species overruled environmental parameters and the bacterial source community in the development of algal-associated BCCs.

All experiments yielded clear separations between free and attached bacteria (Fig. 5, 6A and B, 8, Table 6). A separation between free and attached bacteria could not be proven by Jasti et al., (2005) and Hold et al., (2001), but was confirmed in several other studies (Fandino et al., 2001; Grossart et al., 2005; Sapp et al., 2007a; Rösel et al., 2012). However, due to differences in separation methodology, an underestimation of dissimilarities between both fractions is likely (Rooney-Varga et al., 2005; Allgaier and Grossart, 2006b).

Effects of changes in environmental conditions on alga-associated BCC

The exposure within a natural *M. verticillatum* stand (exp. 1) did not result in any change in BCC (attached or free) of both xenic algal species (Fig. 5). *M. verticillatum* is known to excrete polyphenolic substances with algicidal and bactericidal properties (Hilt et al., 2006; Bauer et al., 2009; 2012), and was thus expected to potentially affect BCC in the vicinity of

the algae. This may either be explained by a lack of bactericidal effects of the lake water, or by parallel algicidal effects increasing the excretion of algal-specific compounds, and thus supporting a specific BCC rather than altering it. However, controls from the macrophyte stand and the macrophyte-free site in exp. 3 also showed no significant differences in BCC (ANOSIM, R = 0.5, p = 0.333). The excretion of allelochemicals in Lake Krumme Laake may therefore be too weak to cause shifts in BCC. Nevertheless, a replacement of laboratory bacteria by bacteria adapted to the bactericidal *Myriophyllum*-derived polyphenols or humic substances (e.g., Steinberg, 2003) present in the lake (concentrations of humic substances: 8.94 ± 0.12 mg L^{-1} in 2007) was expected, but not observed. Thus, changes in other environmental conditions such as pH, temperature (especially diurnal variations), dissolved organic matter quantity and quality and solar radiation (Table 5) were likewise not drastic enough to change the established BCC in either of the tested algal cultures. In this respect, our study contrasts with several previous studies which found specific parameters such as temperature, pH and alkalinity (Crump et al., 2003; Kent et al., 2004; Allgaier and Grossart, 2006b; Kampe et al., 2010; Rösel et al., 2012) to be important drivers of BCC. These studies primarily focused on the BCC of planktonic bacteria, and hence their results may not apply to changes in the community composition of bacteria associated to algae. Our own results agree with those of Sapp et al. (2007a), who found that changes in environmental conditions were not able to markedly influence the BCC of distinct algal cultures.

On the other hand, changes in temperature strongly affected the BCC of *Microcystis* sp. when incubated in dialysis bags in different lakes (Dziallas and Grossart, 2011), and of diatom cultures incubated in the laboratory (Grossart, 1999). One possible explanation for the significant changes in BCC due to environmental conditions observed by Dziallas and Grossart (2011) could be the use of axenic cultures. Bacteria already associated to algae may have an advantage over the surrounding bacterial community, as suggested by differences in the BCC of initially axenic and xenic *D. armatus* cultures exposed in Lake Krumme Laake (exp. 1 and 3, Fig. 9A and B, Table 6). Indeed, Dziallas and Grossart (2011) found no competition between already associated bacteria and the source community, which might lead to overestimations of changes in BCC.

Strong changes in environmental conditions, such as high concentrations of an allelochemical (TA) in exp. 2, significantly affected the BCC of both algal species (Fig. 6A and B). Chosen TA concentrations of 24.5 µmol L^{-1} in exp. 2 were higher than natural concentrations (up to 3.6 µmol L^{-1}; Gross et al., 1996). However, we added TA only once instead of performing continuous additions, and single additions need to be factorized in order to estimate chronic

exposure effects (Nakai et al., 1999; Ahlers et al., 2006). Still, our laboratory approach might overestimate the *in situ* effects of allelochemicals on BCC. Since changes in BCC in our study were accompanied by drastically decreased algal growth rates and increased bacterial abundances (Fig. 7) it is likely that algal mortality may have favoured a specific bacterial community. TA is known for its algicidal (e.g., Bauer et al., 2010) and bactericidal effects (Chung et al., 1998; Akiyama et al., 2001; Walenciak et al., 2002), which both have the potential to affect BCC. Slowly dividing algae have a greater probability of encountering bacteria (Vaque et al., 1989), and algal senescence increases bacterial abundances (Grossart et al., 2005). Higher bacterial abundances in TA treatments can thus be explained by a much lower growth rate of *D. armatus* and the senescence of *S. minutulus* (Fig. 7A and C). In addition, the direct effects of TA on BCC are supported by the fact that gram-positive bacteria and bacteria from the CF cluster seem to be especially inhibited by polyphenols, whereas polyphenols have little or no effects on *α*- and *γ-Proteobacteria* (abundant groups in the present study, Fig. 10; Walenciak et al., 2002). Several aquatic bacterial isolates were even capable of growing with TA as the sole carbon and energy source (Müller et al., 2007), suggesting that bacteria have the potential to degrade and detoxify TA.

Growth rates of *D. armatus* were not negatively influenced when exposed in the lake (exp. 1), and our cultures grew exponentially (lake: $\mu = 0.74 \pm 0.13$ d^{-1}, laboratory: $\mu = 0.58 \pm 0.11$ d^{-1}). Even though *S. minutulus* did not grow exponentially in the lake ($\mu = 0.1 \pm 0.05$ d^{-1}), shifts in BCC were likewise not significant (Fig. 5). Whereas several previous studies have indicated that BCC is strongly correlated to algal growth phases (Riemann et al., 2000; Grossart et al., 2005), minor shifts in BCC following changes in growth rates have also been found for diatoms (Sapp et al., 2007a), dinoflagellates (Hold et al., 2001) and other phytoplankton (Jasti et al., 2005). In our study, changes in growth rates seem to have a negligible effect, in particular on the BCC associated with *S. minutulus*. The importance of species-specific bacteria-alga associations may vary between algal species (this study, Sapp et al., 2007a), and with that, the potential of environmental conditions to controlling alga-associated BCC. The degree to which the BCC of a particular alga is impacted by environmental changes is therefore likely to be specific to each algal species.

Here, we conclude that moderate *in situ* concentrations of polyphenols together with changes of environmental conditions (associated with the transfer from the laboratory to a lake) did not change the BCC associated with the tested algae. On the contrary, drastic changes such as high concentrations of TA dramatically altered BCC by directly and indirectly affecting the algae and their associated bacteria.

Effects of bacterial source community on alga-associated BCC

In our study, changing the bacterial source community by incubating xenic algae in a lake (exp. 1) or by adding lake water to xenic algal cultures in the laboratory (exp. 2) resulted in only minor changes in the BCC of both tested algal species (Fig. 5 and 6). In both experiments, lake bacteria might have been outcompeted by laboratory bacteria associated with the algal surfaces prior to exposure (e.g., Vaque et al., 1989). This is surprising, as bacterial source communities often seem to overrule the effect of environmental conditions with respect to planktonic BCC (Langenheder et al., 2006; Dziallas and Grossart, 2011).

Also, other studies show that bacteria inocula were not able to change the BCC of several marine and freshwater algal species (Schäfer et al., 2002; Jasti et al., 2005). Since environmental conditions also structure bacterial source communities (Lindström et al., 2005; Kampe et al., 2010), a clear separation between these two variables remains difficult.

Algal sensitivities towards allelochemicals modulated by associated bacteria

The obtained bacterial sequences included many different bacterial groups which have previously been found in association with algae (Schäfer et al., 2002; Grossart et al., 2005; Kaczmarska et al., 2005; Sapp et al., 2007b), and which prefer freshwater conditions (Nold and Zwart, 1998; Glöckner et al., 1999; Allgaier and Grossart, 2006b). Due to problems with reamplifying DNA from DGGE bands, our obtained sequences included only a small section of the total bacterial diversity associated with the tested algae. Consequently, we cannot draw conclusions on differences in bacterial associations between *S. minutulus* and *D. armatus*.

Due to the abundant presence of potential polyphenol degraders, a degradation and thus inactivation of polyphenolic allelochemicals by the associated bacteria for both investigated algal species is likely (Fig. 10). These bacteria were not only present in incubation experiments with allelochemicals (exp. 1 and 2), but also in laboratory cultures, pointing to a species-specific association (Fig. 10). Previous studies showed that *D. armatus* is a rather insensitive species towards allelochemicals, whereas *S. minutulus* is known to be highly sensitive (Körner and Nicklisch, 2002; Hilt et al., 2006; but see also Hilt et al., 2012). This is corroborated by the present study showing that growth rates of *S. minutulus* are significantly more inhibited by TA than those of *D. armatus* (MWU, $p = 0.05$). A significant contribution of a distinct, species-specifically associated bacterial community (including potential polyphenol degraders) to this difference in the sensitivities of *D. armatus* and *S. minutulus* towards allelochemicals could not be experimentally shown.

Conclusions

The influence of the algal host overrules effects of changes in environmental conditions such as moderate polyphenol concentrations, light and temperature variation, and bacterial source community as factors for the BCC development. A contribution of this highly alga-specific BCC to differences in sensitivities of each algal species towards allelochemicals, however, could not be shown.

Our results therefore support the "niche" hypothesis (Hardin, 1960): if one algal species offers defined, stable conditions over a long period of time, the same well-adapted bacterial species will outcompete when present. Species-specific associations of bacteria to algae may depend on various factors such as algal extracellular products (Bell and Mitchell, 1972; Bell et al., 1974; Schäfer et al., 2002; Šimek et al., 2011), surface structures and components (Dang and Lovell, 2000; Jasti et al., 2005), viral lyses (Šimek et al., 2011), life history, cellular storage products, antibiotic production (Jasti et al., 2005) and protistan grazing (Muylaert et al., 2002; Šimek et al., 2011). From our experiments, it cannot be concluded which of these factors are the main drivers for niche separation.

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Chapter IV:

Do macrophytes support harmful cyanobacteria? Interactions with a green alga reverse the inhibiting effects of macrophyte allelochemicals on *Microcystis aeruginosa*

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Abstract

Plant-released allelopathic substances are increasingly suggested as potential natural measure to control cyanobacteria blooms in lakes. We tested whether the common cyanobacterium *Microcystis aeruginosa*, known to be sensitive to allelochemicals, remains suppressed when interacting with a less sensitive green alga. We investigated the effects of polyphenolic allelochemicals on single and mixed cultures of the common green alga *Desmodesmus armatus* and *Microcystis aeruginosa* with three different inoculum compositions. Polyphenols were added as tannic acid or released by a macrophyte (*Myriophyllum verticillatum* L.) in coexistence experiments with dialysis bags. Interaction with the green alga turned the inhibiting effect of allelochemicals on the cyanobacterium into an enhancement resulting in increased growth rates. The ratio between *M. aeruginosa* and the green alga consequently became significantly higher in treatments with allelochemicals as compared to controls after three days. When initial growth rates of *M. aeruginosa* and the green alga were similar, this resulted in an increasing abundance of the cyanobacterium under allelochemical presence. Interactions between phytoplankton species thus need to be taken into account when investigating the ecological relevance of macrophyte allelopathy and its potential to suppress cyanobacteria blooms.

Introduction

Cyanobacteria dominance in lakes is a persistent topic in freshwater ecology due to harmful bloom formations associated with eutrophication (e.g., Heisler et al., 2008; O'Neil et al., 2012). Lowering nutrient loading from the catchment and applying in-lake methods to reduce internal loading can decrease the availability of phosphorus, which is a primary cause of cyanobacterial blooms (Hupfer and Hilt, 2008). Sufficient reduction of nutrient concentrations, however, often requires long-term and expensive restoration measures (Hupfer and Hilt, 2008) and toxic substances cannot directly be applied due to their negative consequences for non-target aquatic organisms and human health (e.g., Lam et al., 1995). Re-oligotrophication of lakes during the past 30 years has resulted in a lowered occurrance of cyanobacteria in deep lakes, yet no significant change was observed for cyanobacteria in shallow lakes (Jeppesen et al., 2005). In addition, the effects of climatic change are expected to further enhance the expansion of such cyanobacterial blooms (e.g., Paerl and Huisman, 2008), especially in temperate shallow lakes (Mooij et al., 2007). There thus remains a need for research into the factors controlling cyanobacteria blooms particularly in shallow lakes. Such lakes are known to experience negative feedbacks between macrophyte and phytoplankton, which ultimately result in the occurrence of alternative stable regimes in which either submerged macrophytes or phytoplankton dominate (Scheffer et al., 1993).

One mechanism potentially contributing to the stabilization of clear water conditions in shallow lakes is the excretion of allelopathic substances by submerged macrophytes, suppressing phytoplankton growth (Hilt and Gross, 2008). An increasing number of studies have aimed at identifying allelopathically active macrophyte species that may be suitable as control measure for cyanobacteria production in hypertrophic lakes (e.g., Nakai et al., 1999; Joo et al., 2007; Nam et al., 2008; Zhang et al., 2009) or detecting natural allelochemicals with the potential to develop effective biological "algicides" against harmful cyanobacteria blooms (e.g., Nakai et al., 2000; Zhang et al., 2010).

Indeed, a number of common submerged macrophyte species have been shown to significantly suppress numerous planktonic cyanobacteria species (Hilt, 2006). One of the most common (with a worldwide distribution) and harmful bloom-forming cyanobacterial species is *Microcystis aeruginosa* (e.g., Fogg, 1969; Figueirdo et al., 2004). It can completely dominate the summer phytoplankton in lakes (e.g., Liu et al., 2011). Future eutrophication and climatic warming are expected to synergistically promote the growth of toxic *Microcystis* populations, leading to blooms with higher microcystin content (Davis et al., 2009). Numerous submerged macrophyte species such as *Cabomba caroliniana, Ceratophyllum*

demersum, Eleocharis acicularis, Egeria densa, Limnophila sessiliflora, Potamogeton oxyphyllus and *Vallisneria denseserrulata* (Nakai et al., 1999), *Stratiotes aloides* (Mulderij et al., 2007), *Chara vulgaris* (Zhang et al., 2009) or *Potamogeton pusillus* (Takeda et al., 2011) were found to inhibit *M. aeruginosa* allelopathically. However, most studies have been carried out on the effects of the Eurasian watermilfoil *Myriophyllum spicatum* (Nakai et al., 1999; 2000; Körner and Nicklisch, 2002; Nam et al., 2008; Zhu et al., 2010) or phenolic acids and polyphenols present in this species (Nakai et al., 2000; Dziga et al., 2007; Shao et al., 2009; Zhang et al., 2010). These polyphenols are known to inhibit the activity of photosystem II (PS II) (Körner and Nicklisch, 2002; Leu et al., 2002).

In most studies on the effects of macrophyte allelochemicals on phytoplankton single-species cultures have been used. In these studies, cyanobacteria have often proven to be more susceptible to macrophyte allelochemicals than other phytoplankton groups such as green algae (Hilt and Gross, 2008). Natural phytoplankton assemblages, however, usually contain a high number of species from different phytoplankton groups that compete for nutrients and light (Kayser, 1979). In addition to resource competition, other factors may influence interactions between phytoplankton species and thus their bloom sequence in lakes. These include extracellular metabolites that have been suggested to play a major role in bloom sequence determination (e.g., Keating, 1977). Numerous cyanobacteria and algae release chemicals that inhibit the growth of co-occurring phytoplankton species (e.g., Graneli et al., 2008). In general, the stress induced by the presence of other phytoplankton species may thus affect the species-specific response towards macrophyte allelochemicals. We hypothesized that *M. aeruginosa*, which is known to be sensitive to polyphenolic allelochemicals in single-species cultures, will also be suppressed in a mixed culture with the addition of a less-sensitive phytoplankton species.

To test this we measured the effect of polyphenolic allelochemicals on single-species and mixed cultures of *M. aeruginosa* and the green alga *Desmodesmus armatus* (R.Chodat) E. Hegewald in short-term (3 days) experiments under nutrient saturation. *D. armatus* was chosen based on its known low sensitivity towards polyphenolic allelochemicals (Körner and Nicklisch, 2002; Hilt et al., 2006) and because species of *Scenedesmus sensu lato* (recently split into *Scenedesmus* and *Desmodesmus*) are one of the most common phytoplankton constituents in shallow eutrophic freshwater lakes (Vanormelingen et al., 2009). We compared two methods of allelochemical addition: single additions of the known, commercially available polyphenolic allelochemical tannic acid and dialysis bag coexistence

experiments with the polyphenol-excreting submerged macrophyte *M. verticillatum* (Hilt et al., 2006).

Material and methods

Organisms and culture conditions

Cultures of the green alga *Desmodesmus armatus* (SAG 276-4e) and the cyanobacterium *Microcystis aeruginosa* (SAG 46.80) were obtained from the Culture Collection of Algae (SAG) at the University of Göttingen. Cultures were kept in a modif ed M III nutrient solution (Körner and Nicklisch, 2002), which was also used for all experiments. The nutrients (400 μmol L^{-1} Si, 500 μmol L^{-1} N, 50 μmol L^{-1} P, 10 μmol L^{-1} Fe) were assumed to be non-limiting at the biomass densities used in our experiments (maximum final concentration < 300 μg chl a L^{-1} without conversion). Cultures were grown semi-continuously in a climate-controlled room at 25°C in continuous light of 80 µmol quanta m^{-2} s^{-1}, in a 12:12 h light–dark cycle and shaken manually twice daily. During this phase, cultures were diluted to a fixed chlorophyll *a* (chl *a*) concentration of about 10 µg L^{-1} every day (turbidostat principle). Whorled watermilfoil *Myriophyllum verticillatum* L. was chosen as a submerged macrophyte species with a known high content and release rate of polyphenols with allelopathic effects on algae and cyanobacteria (Hilt et al., 2006; Bauer et al., 2009). Plant shoots (top 15 cm) of *M. verticillatum* were collected in Lake Krumme Laake (Berlin, Germany, 52º25.000'N, 13º 41.150'E) in August 2010 (approximately two months before the experiments) and were maintained rooted in aquaria that contained sediments and tap water until the experiments started.

Experimental design

Two competition experiments were executed, each using three different mixtures (initial compositions): 75% *M. aeruginosa* with 25% *D. armatus*, 50% *M. aeruginosa* with 50% *D. armatus* and 25% *M. aeruginosa* with 75% *D. armatus* based on their chl *a* content (see below). These mixtures were paralleled by treatments consisting of populations of solely *M. aeruginosa* (100% *M.a.*) or *D. armatus* (100% *D.a.*). Test conditions were the same as growth conditions.

In the first experiment (exp. 1), the effect of tannic acid (TA) on single and mixed cultures of *D. armatus* and *M. aeruginosa* was examined at concentrations of 1 mg $L^{-1}$and 5 mg L^{-1}. Preliminary short-term (24 hours) standard toxicity assays performed with TA concentrations ranging from 0.1 to 50 mg L^{-1} had shown that 1 and 5 mg L^{-1} TA decreased growth rates of *D.*

armatus and *M. aeruginosa* without complete inhibition. In addition, these TA concentrations were assumed to be ecologically relevant and comparable to polyphenol concentrations excreted by *M. verticillatum* in exp. 2 (see below). Fifty mL of the cultures were filled into 100 mL f asks and freshly prepared TA stock solution (250 mg TA L^{-1}) was added (0.2 mL and 1 mL to reach a final concentration of 1 mg L^{-1} and 5 mg L^{-1}, respectively) at the start of the experiment. Four replicates were run for the controls, the 1 mg L^{-1} TA and the 5 mg L^{-1} TA treatments, respectively. Chl *a* concentrations were determined each day at the same time and growth rates μ were calculated for day 0-3 as described below. Initial chl *a* concentrations (all data in μg L^{-1}) were 59.1±0.5 in 100% *M. aeruginosa* (10.1±1.3x10^5 cells mL^{-1}), 49.5±0.3 + 26.3±0.6 in 75% *M. aeruginosa* + 25% *D. armatus*, 27.7±0.2 + 36.4±1.1 in 50% *M. aeruginosa* + 50% *D. armatus*, 17.2±0.2 + 48.0±1.0 in 25% *M. aeruginosa* + 75% *D. armatus* and 53.4±0.7 in 100% *D. armatus* cultures (12.5±0.3x10^5 cells mL^{-1}). Initial chl *a* ratios between *M. aeruginosa* and *D. armatus* in the three mixed cultures were thus: 1.88±0.03, 0.76±0.003 and 0.36±0.003, respectively.

In the second experiment (exp. 2), the effect of allelochemicals exuded by *M. verticillatum* on single and mixed cultures of *D. armatus* and *M. aeruginosa* was examined by coexistence tests placing phytoplankton in dialysis bags into flasks containing macrophyte shoots. One day before starting the experiments, *M. verticillatum* shoots were rinsed carefully with tap water to remove attached algae without damaging them and fresh weight (fw) was determined after blotting. Two shoots of *M. verticillatum* (~2.5-3.5 g fw) were placed into 500 mL Erlenmeyer flasks containing 450 mL of modif ed MIII solution, resulting in macrophyte densities (5-7 g fw L^{-1}= 0.35-0.7 g dry weight (dw) L^{-1}) comparable with those occurring in lakes (Grace and Wetzel, 1978). These plants were estimated to exude up to 0.7 mg L^{-1} polyphenols in three days based on measurements by Gross et al. (1996) using *M. spicatum* that exuded 2-4 mg polyphenols g^{-1} dw in 10 days. Plastic plants of similar shape and density (to simulate the shading effect) were used as controls. Fifty mL pure or mixed cultures were poured into sterile dialysis membrane tubes (Wienie-Pak Skinless Sausage Casings, Devro Teepak, Scarborough, Ontario, Canada) with a molecular weight cutoff of 7000. Membranes were washed with distilled water, knotted at one end, and secured at the other end over a glass tube. Chl *a* concentrations were determined each day at the same time and growth rates μ calculated for day 0-3 as described below. Initial chl *a* concentrations (all data in μg L^{-1}) were 63.3±0.19 in 100% *M. aeruginosa* (14±0.4x10^5 cells mL^{-1}), 45.6±0.04 + 31.5±0.4 in 75% *M. aeruginosa* + 25% *D. armatus*, 29.7±0.07 + 43.6±0.29 in 50% *M. aeruginosa* + 50% *D. armatus*, 22.6±0.26 + 58.7±0.15 in 25% *M. aeruginosa* + 75% *D. armatus* and 59.5±1.1 in

100% *D. armatus* cultures ($4.7\pm0.1x10^5$ cells mL^{-1}). Initial chl *a* ratios between *M. aeruginosa* and *D. armatus* in the three mixed cultures were thus: 1.44±0.02, 0.68±0.004 and 0.38±0.004, respectively. These ratios (also in exp. 1) deviated slightly from the initial planning (3, 1, 0.33), but still represent the intended scheme. Figure legends were thus kept for simplicity.

Concentrations of soluble reactive phosphorus (SRP) and nitrate (NO_3^--N) were determined in filtered culture solution at the end of the experiment according to standard methods (Anonymous, 2005).

Determination of growth rates

Growth rates μ of *M. aeruginosa* and *D. armatus* were determined based on measurements of concentrations of chl *a* at the beginning (day 0) and end (day 3) of the experiments as $\mu=\ln(n_3/n_0)$/number of days with n_3 and n_0 being the chl *a* concentration at day 3 and 0, respectively. Chl *a* concentrations were determined from measurements of F_0 fluorescence with a Phyto-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) after dark adaptation for 20 minutes, as F_0 is closely correlated to chl *a* content (Schreiber and Bilger, 1993). Background fluorescence of the nutrient solution and soluble organic compounds was determined from a filtrate (a 0.2 μm filter retained cyanobacteria and algae) and digitally suppressed by the zero-offset function. The Phyto-PAM fluorometer uses four different excitation wavelengths, which allows a separation between cyanobacteria, green algae and diatoms (Kolbowski and Schreiber, 1995). The Phyto-PAM deconvolution reference curve was calibrated for *D. armatus*, whereas the cyanobacteria default setting was used for *M. aeruginosa*. Chl *a* concentrations calculated by the fluorometer software (which uses a standard value) were multiplied by 1.37 and 4.51 for *M. aeruginosa* and *D. armatus*, respectively (conversion factors determined from HPLC measurements of chl *a* of laboratory cultures; for details see Körner and Nicklisch (2002)). Using fluorescence-based chl *a* as a measure for biomass requires constant chl/F_0-ratios (Jakob et al., 2005). These were confirmed by Schmitt-Jansen & Altenburger (2007) in up to 26 day-old periphyton containing diatoms, green algae and cyanobacteria. Growth rates of pure cultures of *M. aeruginosa* and *D. armatus* differed between the two experiments, which allowed the test of two scenarios: lower (exp. 1) and equal (exp. 2) growth rates in controls of *M. aeruginosa* as compared to *D. armatus*.

In exp. 1, additional measurements of particle number and biovolume were performed using a CASY Counter (Schärfesystem, Germany). These data are supposed to be less suitable for a

correct measurement of biomass and growth rates than chl fluorescence, as *D. armatus* can occur in single, two-, four- or eight-celled coenobia and *M. aeruginosa* cells may aggregate into colonies which are difficult to dissociate for counting with a particle counter (no ultrosonication was used). In addition, particle counting does not allow for the distinction of species within mixtures (see Lürling and Roessink, 2006). However, we calculated growth rates based on particle counts and biovolume (here termed particle- and biovolume-based growth rates, method as described above) and checked for correlations between these non-fluorescence based parameters and fluorescence-based growth rates based on total chl *a* (sum of chl *a* of *M. aeruginosa* and *D. armatus*).

Data analysis

In exp. 1, linear regressions were calculated between growth rates of fluorescence- (total chl *a*) and non-fluorescence-based parameters (particle count, biovolume) of the different treatments for the whole experimental period (day 0-3). Particle-, biovolume-, and total chl *a*-based growth rates for days 0-3 in exp. 1 were compared by two-way analyses of variance (ANOVA) with initial composition and treatment as fixed factors. Differences between means were distinguished by Tukey's post hoc comparison ($p < 0.05$). The same procedure was applied for chl *a*-based growth rates of each single species in exp. 1 and 2. When interactions between the initial composition and treatment were significant, a one-way ANOVA with subsequent Tukey's post hoc comparison was adopted comparing the growth rates between the different initial compositions separately for each treatment and species (*M. aeruginosa, D. armatus*) and between the different treatments separately for each initial composition (only exp. 1). The significance of differences between growth rates of control and *M. verticillatum* treatments in exp. 2 was tested by t-tests. The difference between ratios of chl *a* of *M. aeruginosa* and *D. armatus* in mixed cultures of controls and the 5 mg L^{-1} TA (exp. 1) or *M. verticillatum* (exp. 2) treatment at the end of the experiment was tested by t-tests. The percentage inhibition of chl *a*-based growth rates between controls and 5 mg L^{-1} TA (exp. 1) or *M. verticillatum* (exp. 2) treatments was calculated and compared between different initial compositions by one-way ANOVA and subsequent Tukey's post hoc comparison. All analyses were carried out in the statistical package SPSS 17.0 for Windows (SPSS, Chicago, IL, USA).

Results

Effects of TA addition

In exp. 1, growth rates of pure *M. aeruginosa* and *D. armatus* cultures and the three mixtures based on total chl *a* (Fig. 11C) were significantly correlated with those based on particle counts (Fig. 11A) and biovolume (Fig. 11B) for controls and both TA treatments (Table 7).

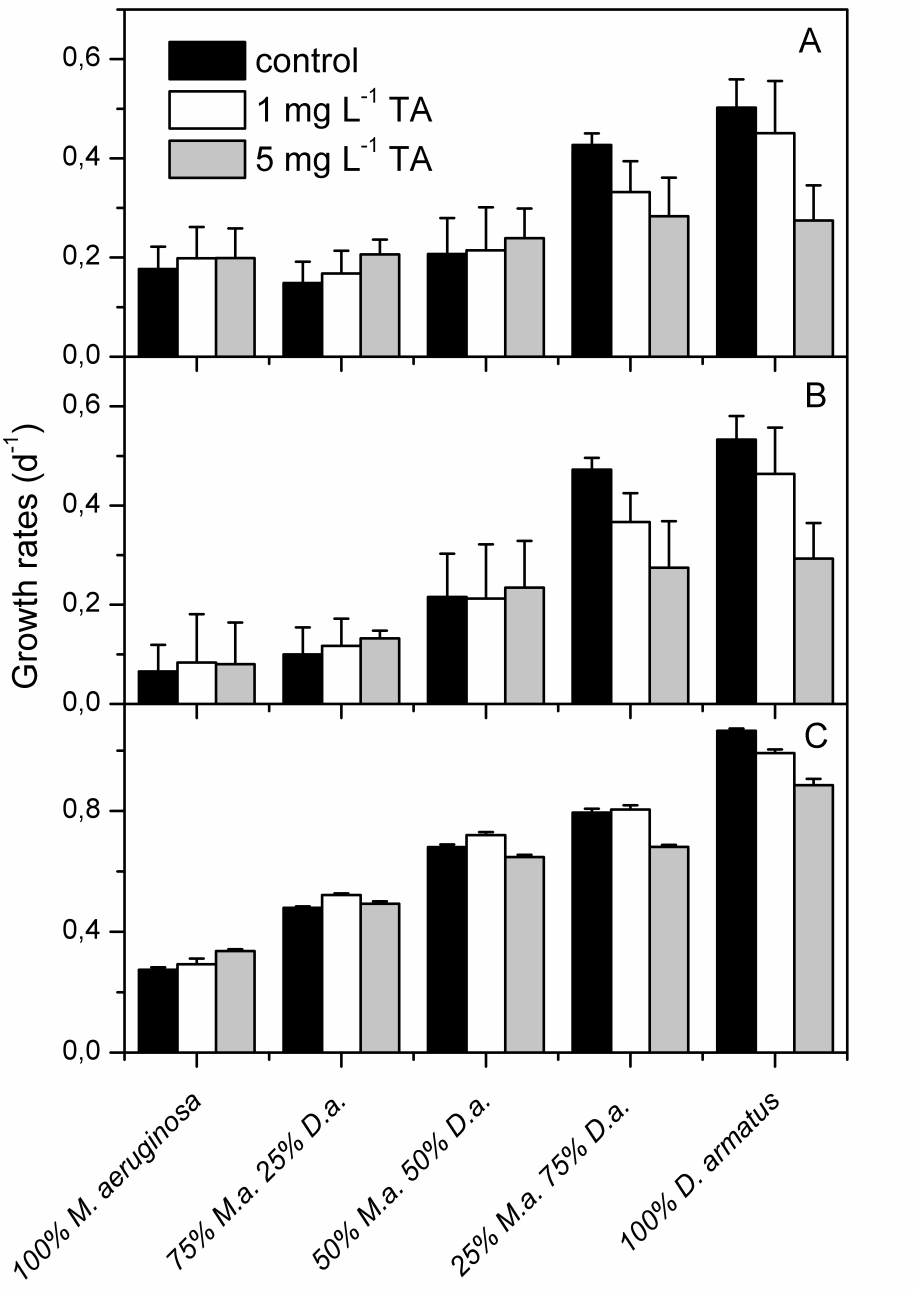

Figure 11:

Particle- (A), volume- (B) and total chl *a*-based (C) growth rates of *Microcystis aeruginosa* (M.a.) and *Desmodesmus armatus* (D.a.) as well as three mixtures with different initial composition (based on chl *a*) grown for 3 days in the absence (black bars; control) and presence of 1 and 5 mg L^{-1} tannic acid (TA) (white and grey bars, respectively). Error bars show standard errors (n = 4), results of linear regressions and two-way ANOVA are given in Table 7 and 8.

Table 7: Linear regression between growth rates of the two pure cultures and three mixtures of *M. aeruginosa* and *D. armatus* (each with 4 replicates thus n=20) based on total chl *a*, particle counts and biovolume grown for 3 days without (control) and with addition of tannic acid (TA) of two different concentrations (Fig. 11A-C).

		slope	R^2	F	p
Control	Total chl *a* – particle count	0.55	0.76	58	<0.0001
	Total chl *a* – biovolume	0.64	0.80	73	<0.0001
1 mg L^{-1}	Total chl *a* – particle count	0.42	0.58	25	<0.0001
TA	Total chl *a* – biovolume	0.65	0.76	58	<0.0001
5 mg L^{-1}	Total chl *a* – particle count	0.4	0.32	8	0.01
TA	Total chl *a* – biovolume	0.48	0.38	11	0.005

Particle- and volume-based growth rates were lower than those based on fluorescence (Fig. 11A-C). Potential reasons for this could be colony and multi-cell coenobia formation in both species, resulting in an underestimation of particle numbers and biovolume by the particle counter (as we did not disintegrate our samples prior to measurements). Particle- , biovolume- and total chl *a*-based growth rates in controls increased significantly in cultures with an increasing share of *D. armatus* in the initial composition (Fig. 11A-C, Table 8). The addition of TA lowered this increase in particle- and biovolume-based growth rates and differences between initial compositions completely disappeared for particle-based growth rates with 5 mg L^{-1} TA addition (Fig. 11, one-way ANOVA, F=1.5, p=0.24). Significant differences between treatments (control, 1 mg L^{-1} TA and 5 mg L^{-1} TA) only occurred in the initial compositions with a high share of *D. armatus* (75% or 100%) (Fig. 11, one-way ANOVA, F=6.1 and 8.9, p=0.02 and 0.007, respectively).

The fluorescence-based determination of chl *a* allowed a distinction between the response of the growth rates of *M. aeruginosa* and *D. armatus* to different initial compositions and TA addition (Fig. 12A, B). Growth rates of *M. aeruginosa* in controls only changed slightly with no clear pattern associated with an increasing share of *D. armatus* (Fig. 12A, Tables 8, 9), whereas *D. armatus* growth rates where significantly lowered by an increasing share of *M. aeruginosa* (Fig. 12B, Tables 8, 9). When growing alone, the growth rate of the green alga was approximately 1.07 d^{-1}, while in the presence of 75% *M. aeruginosa* it dropped by 34% (Fig. 12B).

Table 8: Results of two-way ANOVA comparing growth rates based on particle number, biovolume and total chl *a* of mixed cultures containing *Microcystis aeruginosa* (*M.a.*) and *Desmodesmus armatus* (*D.a.*) and growth rates based on chl *a* of each single species with initial composition and treatment as fixed factors.

Exp. No.	Growth rates based on		df	F	*p*
1	Particle number	Treatment	2	3.4	0.042
	(Fig. 11A)	Composition	4	32.1	<0.001
		Treat. x comp.	8	4.3	0.001
	Biovolume	Treatment	2	5.1	0.010
	(Fig. 11B)	Composition	4	51.9	<0.001
		Treat. x comp.	8	3.3	0.004
	Total chl *a*	Treatment	2	38.9	<0.001
	(Fig. 11C)	Composition	4	1588	<0.001
		Treat. x comp.	8	21.4	<0.001
	Chl *a M.a.*	Treatment	2	37	<0.001
	(Fig. 12A)	Composition	3	28.2	<0.001
		Treat. x comp.	5	5.6	0.001
	Chl *a D.a.*	Treatment	2	36.7	<0.001
	(Fig. 12B)	Composition	3	140	<0.001
		Treat. x comp.	5	3.0	0.023
2	Chl *a M.a.*	Treatment	1	14	0.001
	(Fig. 15A)	Composition	3	24	<0.001
		Treat. x comp.	3	27	<0.001
	Chl *a D.a.*	Treatment	1	56	<0.001
	(Fig. 15B)	Composition	3	66	<0.001
		Treat. x comp.	3	0.58	0.63

The response of the chl *a*-based growth rates of *M. aeruginosa* and *D. armatus* to TA addition was significantly different. Growth rates of *M. aeruginosa* significantly increased with TA addition in all cases except for those with the lowest initial share (Fig. 12A, Table 10). Growth rates of *D. armatus* significantly decreased with TA addition except for the mixture with the lowest share of *D. armatus*. This inhibiting effect was most pronounced when *D. armatus* had a high share in the initial composition (Fig. 12B, Table 10).

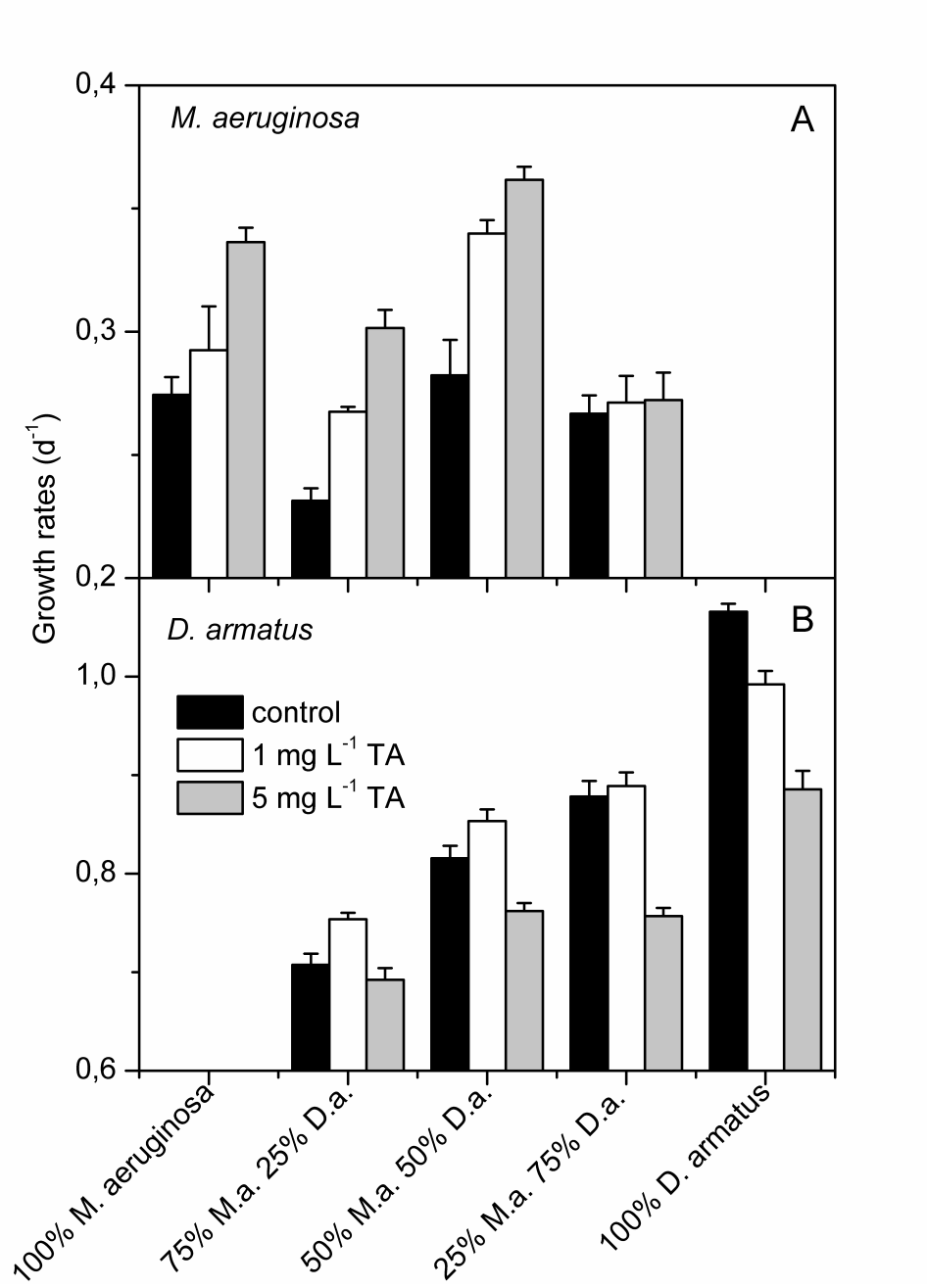

Figure 12:
Chl *a*-based growth rates of *Microcystis aeruginosa* (*M.a.*) (A) and *Desmodesmus armatus* (*D.a.*) (B) as well as three mixtures with different initial compositions during 3 days of growth in the absence (black; control) and presence of 1 and 5 mg L^{-1} tannic acid (TA) (white and grey bars, respectively). Error bars show standard errors (n = 4), statistical differences are given in Tables 8-10.

The ratios between chl *a* of *M. aeruginosa* and *D. armatus* in all three mixtures dropped in controls and TA treatments due to the higher growth rates of *D. armatus* (Fig. 13A). However, as a consequence of the differential response of the cyanobacterium and the green alga to TA addition, the ratio was significantly higher after the addition of 5 mg L^{-1} TA as compared to controls and the 1 mg L^{-1} TA treatment on day 3 (Fig. 13A, one-way ANOVA,

F=23, 52, 20 for 75%, 50% and 25% *M. aeruginosa*, respectively, $p<0.001$ for all). A comparison of the percentage inhibition of chl *a*-based growth rates by TA showed that an increasing share of *D. armatus* has a significantly negative impact (less enhancement) on *M. aeruginosa* whereas a higher share of *M. aeruginosa* promotes *D. armatus* (less inhibition) (Fig. 14A, Table 9).

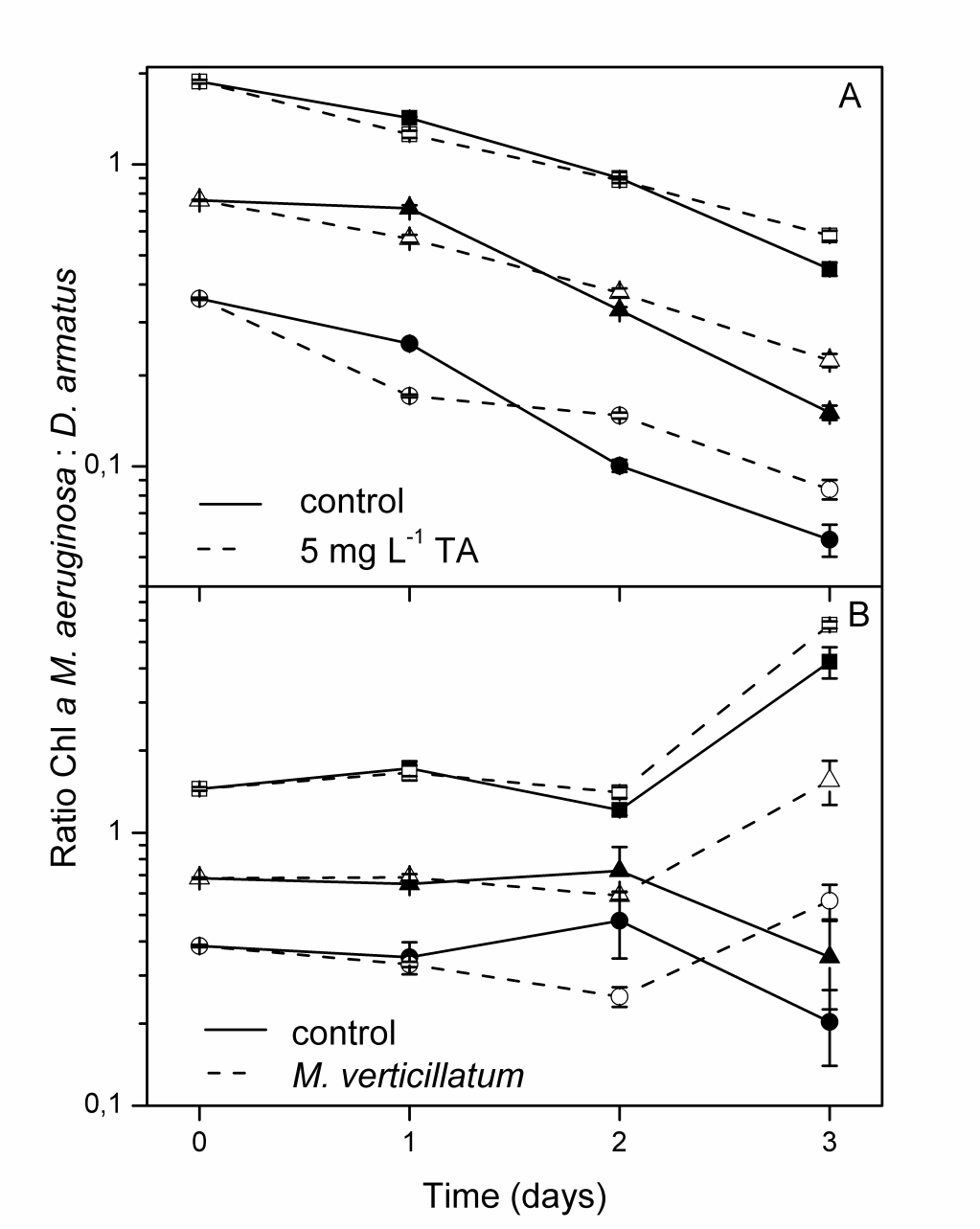

Figure 13:

Ratios of chl *a* of *Microcystis aeruginosa* (*M.a.*) and *Desmodesmus armatus* (*D.a.*) in three mixtures with different initial composition (squares: 75% *M.a.* 25% *D.a.*, triangles: 50% *M.a.* 50% *D.a.*, circles: 25% *M.a.* 75% *D.a.*), grown for 3 days in the absence (control) and presence of 5 mg L^{-1} tannic acid (TA) (1 mg L^{-1} TA treatment was not significantly different from the control) (A) or *Myriophyllum verticillatum* (B). Error bars show standard errors (n = 4).

Table 9: Results of one-way ANOVA and Tukey's post hoc test comparing growth rates based on particle number, biovolume and total chl *a* of mixed cultures containing *Microcystis aeruginosa* (*M.a.*) and *Desmodesmus armatus* (*D.a.*) with different initial composition for control, 1 mg L^{-1} TA and 5 mg L^{-1} TA (exp. 1) and for control and *M. verticillatum* (exp. 2) separately, as well as growth rates based on chl *a* of each single species and inhibition rates of chl *a*-based growths rates .

Exp. No.	Growth rates based on		F	*p*	100% *M.a.*	75% *M.a.*	50% *M.a.*	25% *M.a.*	0% *M.a.*
1	Chl *a M.a.*	Control	5.9	0.01	b	a	b	ab	
	(Fig. 12A)	1 mg L^{-1} TA	9.5	0.002	a	a	b	a	
		5 mg L^{-1} TA	25.8	<0.001	b	a	b	a	
	Chl *a D.a.*	Control	162	<0.001		a	b	c	d
	(Fig. 12B)	1 mg L^{-1} TA	71	<0.001		a	b	b	c
		5 mg L^{-1} TA	38.8	<0.001		a	b	c	d
1	Inhibition *M.a.*	5 mg L^{-1} TA	219	<0.001	a	a	a	b	
2	(Fig. 14A)	*M. verticillatum*	18.8	<0.001	a	b	d	c	
1	Inhibition *D.a.*	5 mg L^{-1} TA	8.2	0.003		a	a	b	b
2	(Fig. 14B)	*M. verticillatum*	23.2	<0.001		a	b	ab	b
2	Chl *a M.a.*	Control	21.6	<0.001	b	b	a	a	
	(Fig. 15A)	*M. verticillatum*	36	<0.001	a	b	b	a	
	Chl *a D.a.*	Control	19.3	<0.001		a	bc	b	c
	(Fig. 15B)	*M. verticillatum*	71.1	<0.001		a	b	b	c

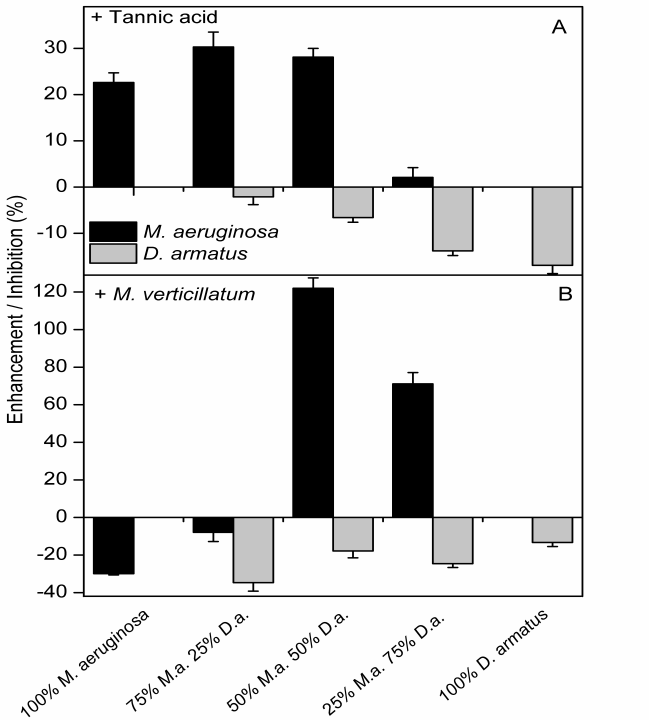

Figure 14:

Enhancement or inhibition of chl *a*-based growth rates of *Microcystis aeruginosa* and *Desmodesmus armatus* as well as three mixtures with different initial compositions grown for 3 days under the influence of 5 mg L^{-1} tannic acid (A) or the presence of *Myriophyllum verticillatum* (B) as compared to controls. Error bars show standard errors (n = 4), statistical differences are given in Table 9.

Table 10: Results of one-way ANOVA and Tukey's post hoc test comparing growth rates based on total chl *a* of mixed cultures containing *Microcystis aeruginosa* (*M.a.*) and *Desmodesmus armatus* (*D.a.*) and growth rates based on chl *a* of each single species between control, 1 mg L^{-1} TA and 5 mg L^{-1} TA (exp. 1) separately for different initial compositions.

Growth rates based on		F	*p*	Control	1 mg L^{-1} TA	5 mg L^{-1} TA
Chl *a M.a.*	100% *M.a.*	7.6	0.012	a	ab	b
(Fig. 12A)	75% *M.a.*	44.1	<0.001	a	b	c
	50% *M.a.*	19.0	0.001	a	b	b
	25% *M.a.*	0.09	0.916	a	a	a
Chl *a D.a.*	25% *D.a.*	0.6	0.004	a	b	a
(Fig. 12B)	50% *D.a.*	20.5	<0.001	b	b	a
	75% *D.a.*	29.7	<0.001	b	b	a
	100% *D.a.*	39.6	<0.001	c	b	a

Effects of *M. verticillatum*

Similar results were obtained in exp. 2, which tested the effect of *M. verticillatum* on mixtures of *M. aeruginosa* and *D. armatus*. Chl *a*-based growth rates of controls of *D. armatus* decreased significantly as the initial share of *M. aeruginosa* increased (Fig. 15B, Table 9).

When growing alone, the growth rate of the green alga was about 0.68 d^{-1}, while in the presence of 75% *M. aeruginosa* it dropped by 43% (Fig. 15B). Contrary to the first experiment, however, growth rates of *M. aeruginosa* were also affected by the presence of *D. armatus*: in controls, they were lower at a higher initial share of *D. armatus* (50% and 75%, Fig. 15A, Table 9). When growing alone, the growth rate of the cyanobacterium was about 0.62 d^{-1}, while in the presence of 75% *D. armatus* it dropped by 50% (Fig. 15A). The generally higher growth rates of *M. aeruginosa* in this experiment as compared to those in exp. 1 might be a potential reason for this difference.

The presence of *M. verticillatum* qualitatively resulted in a similar response as with TA additions: decreased growth rates for *D. armatus* (Fig. 15B). For *M. aeruginosa* increased chl *a*-based growth rates were only detected at lower initial share of 50% and 25%, whereas pure cultures were significantly inhibited by *M. verticillatum* (Fig. 15A).

Comparing the percentage inhibition of chl *a*-based growth rates revealed that the results of exp. 1 are reversed under the influence of *M. verticillatum*: an increasing share of *D. armatus* has a positive impact (turning inhibition into enhancement) on *M. aeruginosa*, whereas a higher share of *M. aeruginosa* results in a higher inhibition of *D. armatus* by *M. verticillatum* (Fig. 14B, Table 9).

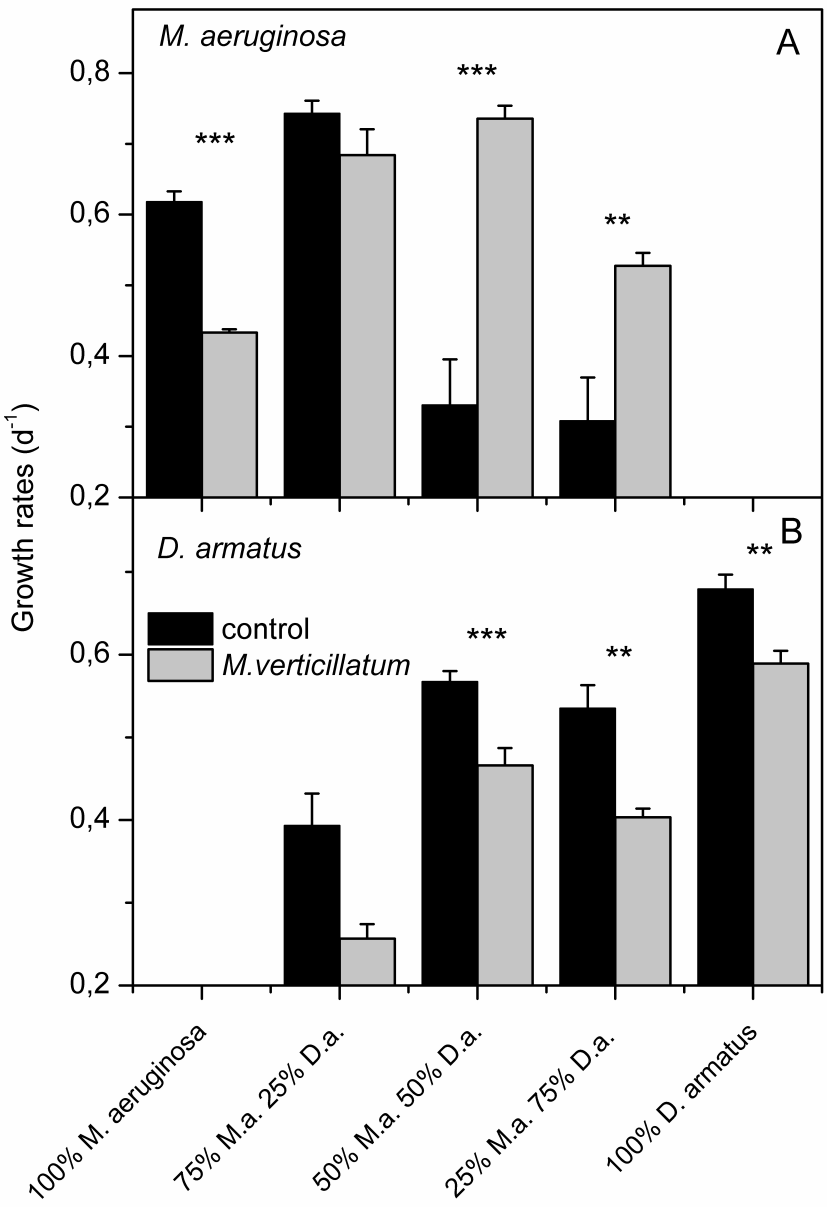

Figure 15:

Chl *a*-based growth rates of *Microcystis aeruginosa* (*M.a.*) (A) and *Desmodesmus armatus* (*D.a.*) (B) as well as three mixtures with different initial compositions calculated for 3 days of growth in the absence (black; control) and presence of *Myriophyllum verticillatum* (grey). Error bars show standard errors (n = 4), differences between control and *M. verticillatum* treatment are indicated by asterisks (*p<0.05, **p<0.01, ***p<0.001), statistical differences between initial compositions are provided in Tables 8-9.

The ratio between *M. aeruginosa* and *D. armatus* chl *a* at the end of the experiment was significantly higher (t-test, p<0.05) in all three mixtures when growing under the influence of *M. verticillatum* as compared to the controls (Fig. 13B). The ratios of both controls and *M. verticillatum* treatments were higher than in exp. 1 due to the higher growth rates of *M. aeruginosa* in exp. 2 as compared to exp. 1. The increase of the ratios during exp. 2 were comparable to those of exp. 1 for 75% and 25% *M. aeruginosa* mixtures, but much higher in the 50% mixture (Fig. 13A, B). An increase in the ratio between *M. aeruginosa* and *D.*

armatus chl *a* at the end of the experiment thus occurred in both tested scenarios: lower and equal growth rates in controls of *M. aeruginosa* as compared to *D. armatus*.

Nutrient concentrations revealed a slightly lower (t-test, p=0.04) SRP concentration in the *M. verticillatum* treatments (0.96 ± 0.02 mg L^{-1}) compared to the controls (1.05 ± 0.03 mg L^{-1}), whereas no differences were detected for NO_3^--N (3.92 ± 0.42 and 4.28 ± 0.36 mg L^{-1}, respectively).

Discussion

Interaction between *M. aeruginosa* and *D. armatus*

When assessing the effect of macrophyte allelochemicals or other substances on phytoplankton mixtures, first the phytoplankton species interactions in controls have to be discussed. In our study, *D. armatus* had no effect on *M. aeruginosa* in the first experiment when growth rates of *M. aeruginosa* were low (Fig. 12A), but inhibited the growth of *M. aeruginosa* in mixed cultures in exp. 2, when growth rates of both species were similar in pure cultures (Fig. 15A). Similar results of a negative impact of a green alga (*Scenedesmus obliquus*) on a cyanobacterium (*M. aeruginosa*) were obtained by Lürling and Roessink (2006) in longer lasting competition experiments. Gregor et al. (2008) detected no significant effect of the green alga *Pseudokirchneriella subcapitata* on the cyanobacterium *Aphanothece clathrata*. On the other hand, *M. aeruginosa* had a negative impact on the growth of *D. armatus* in both experiments of our study (Fig. 12B, 15B). However, Lürling and Roessink (2006) found no negative effect of *M. aeruginosa* on *S. obliquus*, and Franklin et al. (2004) observed an increased growth rate for *P. subcapitata* when in the presence of *M. aeruginosa*.

Resource competition and biotic interactions are potential factors explaining these diverse results. Under laboratory conditions when predation is excluded, phytoplankton species interactions are influenced by the availability of resources such as light (e.g., Mur et al., 1978) and nutrients (e.g., Hyenstrand et al., 2000). We excluded competition for nutrients by high nutrient supply and a short duration of the experiments. Light limitation can probably also be excluded as growth rates calculated for days 0-3 were higher than those calculated for days 0-2 (data not shown). We thus conclude that nutrient and light limitation did not influence the interaction between *M. aeruginosa* and *D. armatus* during this short-term experiment.

In interactions between phytoplankton species, however, temperature also plays a significant role. Our study was performed at 25°C, which is the minimum temperature required for the maximum growth rates of most cyanobacteria (e.g., Paerl and Huisman, 2008). Pure cultures of *M. aeruginosa* still had a lower or equal growth rate (calculated for days 0-3) than those of

D. armatus (means of exp. 1: 0.27 ± 0.01 d^{-1} and 1.07 ± 0.01 d^{-1}, exp. 2: 0.62 ± 0.01 d^{-1} and 0.68 ± 0.02 d^{-1}, respectively). Results should thus be valid for lower temperatures as well, when most common planktonic cyanobacteria achieve lower growth rates than single-celled green algae (at 20 °C and light saturation: 0.3-1.4 versus 1.3-2.3 d^{-1}; Van Liere and Walsby, 1982). Variability in growth rates of controls between experiments was attributed to the use of highly dynamic batch culture systems. Our experiments showed that *M. aeruginosa* increased its share in mixtures with polyphenol addition under both situations, with lower or equal growth rates compared to *D. armatus*.

When resource competition is excluded, reciprocal effects on the growth of phytoplankton species in mixtures might be explained by the release of allelochemicals by algae and cyanobacteria (e.g., Smith and Doan, 1999; Graneli et al., 2008). *M. aeruginosa* was shown to allelopathically inhibit the growth of *Chlorella pyrenoidosa* by linoleic and linolenic acids (Ikawa et al., 1996) and the freshwater dinoflagellate *Peridinium gatunense* (Sukenik et al., 2002). Also Harrass et al. (1985) and Gregor et al. (2008) attributed the negative effects of other cyanobacteria (*Anabaena cylindrica, Aphanothece clathrata*) on green algae (*Scenedesmus obliquus, P. subcapitata*) to the release of allelochemicals. On the other hand, Jia et al. (2008) reported an allelopathic inhibition of *M. aeruginosa* by *Scenedesmus obliquus*. We thus assume that allelopathic effects of both *M. aeruginosa* and *D. armatus* may have been involved in the observed reciprocal inhibitory effects in our mixed cultures. Our experiments, however, were not designed to unravel this interaction and thus lack proper controls for final evidence.

Effect of macrophyte allelochemicals on phytoplankton species interactions

The results of our study clearly demonstrate that allelochemicals released by submerged macrophytes affect the interaction between a green alga (*D. armatus*) and a cyanobacterium (*M. aeruginosa*), favouring the latter. An increasing share of the green alga *D. armatus* turned the inhibitory effect of the allelochemically-active submerged macrophyte *M. verticillatum* on the cyanobacterium *M. aeruginosa* into an enhancement. At the same time, *D. armatus* showed a stronger decline in the presence of *M. verticillatum* when *M. aeruginosa* was added. When tested without interaction (100%), particle-, biovolume-, and chl *a*-based growth rates of *M. aeruginosa* were not affected or even slightly enhanced (chl *a*) by TA in exp. 1, whereas *D. armatus* was significantly inhibited by TA additions in all parameters. This points to a higher sensitivity of *D. armatus* towards TA, but *M. aeruginosa* was only growing slowly in that experiment which may have affected the results. In contrast, pure *M. aeruginosa* was

more sensitive than *D. armatus* towards the impact of *M. verticillatum* (30% versus 13% reduction in chl *a*-based growth rates). In general, cyanobacteria are considered more susceptible to allelochemicals than green algae (Mulderij et al., 2007; Hilt and Gross, 2008; Zhu et al., 2010), although considerable differences have been observed among different cyanobacteria (Nakai et al., 1999; Körner and Nicklisch, 2002) as well as among green algae species (Hilt, 2006; Hilt et al., 2012). Different results were obtained for photosynthesis-inhibiting herbicides, where in general, green algae and diatoms were expected to be more sensitive than cyanobacteria (Peterson et al., 1997; Fairchild et al., 1998). This differential sensitivity, however, also depends on the temperature. At low temperatures the cyanobacterium *Oscillatoria limosa* was twice as sensitive to atrazine as at high temperature (Berard et al., 1999). This temperature-dependent sensitivity could be due to the turnover rates of the D1 protein, which is the specific target of atrazine and which could contribute to the recovery of PS II activity after atrazine binding (Berard et al., 1999). The presence of free and attached bacteria may also have influenced the effect of allelochemicals on *M. aeruginosa* and *D. armatus*. Single-species studies revealed significant positive and negative effects of bacteria on the sensitivity of *D. armatus* to TA (Bauer et al., 2010). As algae may harbour distinct species-specific bacterial communities (e.g., Grossart et al., 2005), the use of xenic cultures is closer to *in situ* conditions and was thus preferred in our study.

Adding inhibiting substances to mixtures of interacting species could lead to the replacement of susceptible species by more resistant ones (e.g., Berard et al., 1999). In our experiments, *M. aeruginosa* was expected to be replaced by *D. armatus* due to its higher sensitivity towards allelochemicals, as established by earlier studies (e.g., Körner & Nicklisch, 2002) and single species tests in exp. 2 (see above). Contrary to this expectation, *M. aeruginosa* was enhanced under the presence of the allelopathically active submerged macrophyte *M. verticillatum* when interacting with *D. armatus*. This was even more surprising given the fact that *M. aeruginosa* was suppressed by the green alga under control conditions without *M. verticillatum*. One important aspect explaining these effects might be the excretion of organic compounds stimulating growth of *M. aeruginosa* by *D. armatus* under the influence of allelochemicals or by *M. verticillatum* itself. More than half of all cyanobacteria have been found to be facultative photoheterotrophs (Stal and Moezelaar, 1997), and Kamjunke and Jähnichen (2000) described leucine incorporation by *M. aeruginosa*. The fact that *M. aeruginosa* growth stimulation in the presence of *M. verticillatum* was only found with higher shares of the green alga (50% and 75%, Fig. 15A) indicates that *D. armatus*, rather than a direct impact of *M. verticillatum* excretion products, was involved. Both, the excretion of

stimulating substances by *D. armatus* under the influence of *M. verticillatum* or the decline of the excretion of *M. aeruginosa*–inhibiting substances by *D. armatus* under the influence of *M. verticillatum*, are possible. Similarly, *M. aeruginosa* possibly released *D. armatus* growth-inhibiting compounds as a result of exposure to *M. verticillatum*.

A complete reversal of the competitive outcome between a cyanobacterium and a green alga has also been reported for the photosynthesis-inhibiting herbicide metribuzin tested on single- and mixed cultures of *M. aeruginosa* and the green alga *Scenedesmus obliquus* (Lürling and Roessink, 2006). Furthermore, an increase in the abundance of cyanobacteria was recorded for natural phytoplankton communities in dialysis bags or outdoor pond mesocosms under the influence of allelopathically active macrophytes (Jasser, 1995), after addition of the diphenyl-ether herbicide fomesafen (Caquet et al., 2005) and in natural periphyton communities after adding of the PS II inhibitor isoproturon (Schmitt-Jansen and Altenburger, 2007).

Conclusions

Our results show that one of the most common harmful freshwater cyanobacteria, *Microcystis aeruginosa*, is inhibited (i) when interacting with a green alga (in short-term experiments) and (ii) by macrophyte allelochemicals when growing in pure culture. However, when both factors (interaction with a green alga and addition of macrophyte allelochemicals) act in parallel, growth inhibition is reversed into enhancement. Allelopathically active macrophytes might thus support cyanobacteria rather than suppress them *in situ*. A simple transfer of our results to field conditions, however, is not possible as our short-term laboratory assays with two phytoplankton species were still far from natural conditions featuring manifold interactions. Our results imply that conclusions concerning the *in situ* growth inhibition potential of macrophyte-released allelochemicals derived by single cyanobacterium species studies should be drawn with caution. Future studies searching for the potential of natural allelochemicals to inhibit toxic cyanobacteria blooms in eutrophic lakes should thus consider interactions of cyanobacteria with other species present in the respective phytoplankton communities as well as other factors influencing the competitive outcome between phytoplankton species such as light and temperature.

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Chapter V:

Sensitivity of the green alga *Pediastrum duplex* Meyen to allelochemicals is strain-specific and not related to co-occurrence with allelopathic macrophytes

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The final version will be available at www.plosone.org

Abstract

Interspecific differences in the response of microalgae to stress have numerous ecological implications. However, little is known of intraspecific sensitivities and the potential role of local genetic adaptation of populations. We compared the allelochemical sensitivity of 23 *Pediastrum duplex* Meyen strains, a common component of the freshwater phytoplankton. In order to test for local genetic adaptation, strains were isolated from water bodies with and without the allelopathically active submerged macrophyte *Myriophyllum*. Strains were assigned to *P. duplex* on the basis of cell shape and colony morphology and only *P. duplex* strains that belonged to the same lineage in an ITS rDNA phylogeny were used. Inhibition of strain growth rates and maximum quantum yields of photoystem II were measured after exposure to tannic acid (TA) and co-culture with *Myriophyllum spicatum*. Growth rate inhibition varied over one order of magnitude between the *P. duplex* strains. There was no correlation between the presence of *Myriophyllum* in the source location and the sensitivity of the strains to TA or the presence of *Myriophyllum*, suggesting that at least strong unidirectional local adaptation to *Myriophyllum* had not taken place in the studied water bodies. The maximum quantum yield of photosystem II of TA exposed algae decreased, whereas the yield of algae exposed to *M. spicatum* was slightly higher than that of the controls. The ranking of *P. duplex* strain sensitivities differed between the types of exposure (single additions of TA versus co-existence with *M. spicatum*) and the parameter measured (growth rate versus maximum quantum yield), emphasizing the importance of measuring multiple traits when analyzing strain-specific sensitivities towards allelochemicals. The observation that sensitivities to allelochemicals vary widely among strains of a single freshwater algal species should be taken into account if evaluating ecological consequences of allelopathic interactions.

Introduction

Natural populations of phytoplankton show high genetic diversity in ecologically important traits (e.g., Fisher et al., 1973; Murphy and Belastock, 1980; Wood ad Leatham, 1992; Behra et al., 1999; Vanormelingen et al., 2009). The majority of phytoplankton studies focus on interspecific-sensitivities to toxicants (Menzel et al., 1970; Fisher et al., 1973; Murphy and Belastock, 1980; Foster, 1982), which can span several orders of magnitude (Jensen et al., 1974). These differences are caused by genetic variability, environmental variability or an interaction between the two (Hoffman and Parsons, 1997) and even in single clonal cultures genetic variability may arise rapidly through *de novo* mutations (e.g., Loxdale and Lushai, 2003; López-Rodas et al., 2007). Often differential natural selection leads to local genetic adaptation of populations to their ambient environment (Fisher et al., 1973; Kawecki and Ebert, 2004; Lakeman et al., 2009). Consequently, the strain origin may form the basis of strain-specific responses. For example, Japanese and Australian strains of *Chatonella marina* have different tolerances to high light intensities, correlating with the water clarity of their origin (Marshall and Newman, 2002). Similarly, neritic diatom strains were found to be less sensitive to polychlorinated biphenyls (PCBs) than oceanic strains of the same species (Fisher et al., 1973). In the latter case, it was proposed that an adaptation occurred, as coastal waters are polluted with PCBs. Because coastal waters offer less stable conditions, it was further suggested that neritic strains should be more stress resistant in general (Fisher et al., 1973; Murphy and Belastock, 1980). However, adaptations specific to a stressor and overall tolerance may or may not occur simultaneously (Murphy and Belastock, 1980).

One of the potentially important ecological traits of phytoplankton is their sensitivity towards allelochemicals. All aquatic primary producers are capable of producing and releasing allelopathically active compounds and numerous cyanobacteria, algae and submerged macrophytes release chemicals that inhibit the growth of co-occurring phytoplankton species (e.g., Gross, 2003). Differences in the response of phytoplankton to allelochemicals have yet mainly been discussed at the species level (e.g., Hilt and Gross, 2008; De Figueiredo et al., 2011). Epiphytic algae and cyanobacteria species were found to be less vulnerable to macrophyte allelochemicals (Hilt, 2006), potentially due to resistance by co-evolution as proposed by Reigosa et al. (1999). Environmental adaptation and co-evolution were previously suggested to decrease the impact of allelopathic interactions and doubts were raised that allelopathy would even occur between plants that have co-evolved (Rabotnov, 1982). Based on these findings, the novel-weapon hypothesis was created (Callaway and Aschehoug, 2000; Callaway and Ridenour, 2004), which proposes that some invasive plants

may perform better in invaded regions because they introduce unique, species-specific, biochemical impacts to native plant and soil microbial communities. The first indications for adaptation of algal populations to allelochemicals were provided by Al-Sheri (2010), who showed a higher sensitivity of a green algal (*Scenedesmus obliquus*) strain to extracts of the allelopathically active macrophyte *Stratiotes aloides,* when they were isolated from macrophyte-free ponds as compared to a strain from a pond with *S. aloides*. However, support for phytoplankton strain-specific sensitivities to allelochemicals and adaptation of populations against plant-released allelochemicals requires a more comprehensive study comparing a larger number of strains from several origins.

In the present study, we compared the sensitivities of 23 strains of *Pediastrum duplex* Meyen (a planktonic green alga common in eutrophic freshwaters) to polyphenolic allelochemicals. Algal strains were isolated from two macrophyte-free lakes (13 strains) and two lakes with stands of allelopathically active macrophytes (*Myriophyllum* spp.). Growth rates and maximum quantum yields of photosystem II of the algal strains were measured after single additions of a synthetic polyphenolic allelochemical (tannic acid, TA), and in co-existence with experiments involving *M. spicatum,* which is known for exudation of polyphenols. We hypothesized that (1) *P. duplex* strains exhibit significantly different sensitivities to allelochemicals, and (2) that sensitivities of strains isolated from lakes with *Myriophyllum* spec. are lower than those of strains from macrophyte-free lakes due to local genetic adaptation.

Materials and Methods

Ethics statement

Myriophyllum spicatum was harvested from Lake Flakensee with permission of the Brandenburg ministry of environment, health and consumer protection. Phytoplankton samples from Lake Müggelsee, Lake Krumme Laake and Lake Teufelssee were taken with permission of the Berlin Senate, department for urban development and environment. Phytoplankton samples from Lake Molenmeers and Kalken were taken with permission of the Belgium NGO Natuurpunt.

Test organisms and culture conditions

Live phytoplankton samples were collected from 4 different ponds or lakes (Table 11), either containing no macrophytes or dense stands of submerged *Myriophyllum spicatum* (pond "Molenmeers") or *M. verticillatum* (lake "Krumme Laake"). Both *Myriophyllum* species are

known to produce and exude polyphenolic allelochemicals affecting several phytoplankton species (Gross et al., 1996; Hilt et al., 2006; Bauer et al., 2009). In Krumme Laake (KL), *M. verticillatum* stands were restricted to one bay, so that additional water samples could be obtained from a macrophyte-free bay (300 m distant to macrophyte stands) to test for intra-lake differences in strain sensitivities. *P. duplex* strains, recognized based on the diagnostic cell shape and presence of intercellular spaces (Komárek and Fott, 1983), were isolated from water samples by micropipetting (Vanormelingen et al., 2007). Cultures were first grown in WC medium (Guillard and Lorenzen, 1972; without pH adjustment or vitamin addition) in well plates at 18 ± 0.5°C and 20-30 µmol photons m^{-2} s^{-1}. For experiments, they were grown in modified (Körner and Nicklisch, 2002) MIII medium (Nicklisch, 1992) at pH 7.9 ± 0.1, 20 ± 0.5°C and 80 µmol photons m^{-2} s^{-1} under 12:12 h light:dark conditions in a conditioning cabinet. Cultures were shaken gently at 60 r.p.m. Strains of some additional *Pediastrum sensu lato* species for phylogenetic comparison were isolated and cultured from the same four ponds as well as from Lake Müggelsee (MUGGEL5, 8, 9, 10, 13, 14, coordinates 52°50´68.44N, 13°42`47.32”E, sampled 15/07/2012).

Table 11: Characteristics of the water bodies used for isolation of *P. duplex* strains

Water body	Molenmeers (Molen)	Krumme Laake (KL)		Teufelssee (Teufel)	Kalken gracht (Kalken)
Coordinates	51°01’57.25”N 3°55’07.61”E	52°43´48.78“N 13°63´50.56”E		52°41`81.44”N 13°68`87.72”E	51°01’38.91”N 3°55’19.05”E
Sampling date	8 July 2010	20 July 2010		20 July 2010	8 July 2010
Isolation date	9 July 2010	24-30 July 2010		24-30 July 2010	9 July 2010
Area (ha)	0.14	3		1.2	3.1
Mean depth (m)	1.5	4		2	2
Presence of macrophytes	*M. spicatum*	*M. verticillatum*	Macrophyte free bay	None	None
P. duplex strain numbers	13, 14, 15, 20, 22	3, 5, 8, 9, 14	N2, N10	4, 6, 8	49, 50, 55, 56, 57, 58, 59, 60

Given the high incidence and diversity of algal (pseudo)cryptic species, including *Pediastrum duplex* (McManus and Lewis, 2011), ITS rDNA sequences were used for assigning *P. duplex* strains to species level. *P. duplex* strains were randomly chosen from within each pond for sequencing, until a total of 50 sequenced *P. duplex* strains was reached. For phylogenetic comparison, the ITS rDNA of 2 *P. duplex* var. *elegans*, 2 *P. boryanum*, 5 *P. tetras*, and 4 *P. angulosum* strains were also sequenced. *Pediastrum* was recently split in five genera (Buchheim et al., 2005); however at least part of this revision is not well-supported (McManus and Lewis, 2011), thus we continue to apply the previous wide genus concept. The DNA sampling and extraction methods, polymerase chain reaction, as well as sequencing were performed as described by Vanormelingen et al. (2007), with the first exception that purification for the PCR product was achieved enzymatically, using Exonuclease I to remove leftover primers and shrimp alkaline phosphatase to remove dNTPs, and the second exception that DITS2 (5'-CGC TGC GTT CTT CAT CGA TG-3') and DITS3 (5'-ACA ACT TTC AGC AAT GGA TGT C-3') were used as sequencing primers (Zechman et al., 1994). All sequences were submitted to GenBank (accession numbers will follow soon). Phylogeny reconstruction was done with Bayesian Inference using MrBayes version 3.1.1 (Ronquist and Huelsenbeck, 2003). The GTR + G + I model was applied with four rate categories. No initial values were assigned to the model parameters. Two runs of four Markov Chains (one cold and three heated) were run for 10 million generations and sampled every 250 generations. This yielded a posterior probability (PP) distribution of 40,001 trees. After exclusion of 20,000 ''burn-in'' trees, PPs were calculated by constructing a 50% majority-rule consensus tree.

Algal concentrations and growth rate

Growth rates of algae were determined based on chlorophyll (chl) fluorescence measured with a MAXI-Imaging-PAM (exp. 1, pulse-amplitude-modulated) or a Phyto-PAM (exp. 2) fluorometer (Fa. Walz, Effeltrich, Germany). Before measurement, the cultures were dark adapted for 15 min. For Phyto-PAM measurements, 2 mL of algal suspension were placed in a cuvette equipped with a magnetic bar and a stamp. Measuring frequency was set to 2 and damping to 3. Maxi-Imaging-PAM measurements were conducted directly in 24-well plates (cellstar, Greiner bio-one, Frickenhausen, Germany) with 2 mL of algal culture. Minimal fluorescence F_0 (Schreiber, 1996) was determined and used as proxy for chl *a* content of the algal cultures (Lürling and Verschoor, 2003). As we did not convert data into real chl *a* values, we subsequently use the term chl F_0. Growth rate μ was calculated as:

$$\mu\ (d^{-1}) = \ln (\text{chl } F_0\,(t_x) - \text{chl } F_0\,(t_0)) / t,$$

where t is time in days, chl F_0 (t_0) is the chl F_0 value at day 0, and chl F_0 (t_x) is the chl F_0 value at day x.

This calculation is valid for exponentially growing cultures consistently measured at the same time of the day (Körner and Nicklisch, 2002).

Maximal quantum yields of the photosystem (PS) II (hereon termed photosynthetic yields) were determined after applying a single saturation pulse (Schreiber, 1996) based on the equation

$Y = (F_v - F_m)/F_m$,

where Y is the maximum quantum yield, F_v is the variable fluorescence (difference between F_0 after dark-adaption and F_m after the saturation pulse), and F_m the maximal fluorescence after the saturation pulse.

Growth rate and photosynthetic yield inhibition by tannic acid (exp. 1)

In the first experiment, we compared the sensitivities of 23 *P. duplex* strains to single additions of tannic acid (TA). TA is a common and commercially available polyphenol present in *Myriophyllum* (Planas et al., 1981) and closely related to the most active compounds in *M. spicatum* (Gross et al., 1996) and *M. verticillatum* (Bauer et al., 2009). Although single additions of allelochemicals cannot provide convincing ecological support for allelopathic interactions, this approach is most often applied in aquatic allelopathy research (Hilt and Gross, 2008). The experiment was conducted in 24-well plates (cellstar, Greiner bio-one, Frickenhausen, Germany) in a conditioning cabinet with light from above (12:12 hour dark:light period with 200 µmol photons m^{-2} s^{-1}) and at 20 ± 0.5 °C. Algal cultures were kept in the exponential growth phase before subjecting them to experiments to assure comparable physiological states. Each well contained 2 mL of algal culture with or without TA and a starting concentration of chl. F_0 = 10 µg L^{-1} (Phyto-PAM measurements). To derive at a final TA concentration of 10 mg L^{-1} (which is comparable to concentrations possible under *in situ* conditions, Gross et al., 1996), 1.8 mL of an 11 mg L^{-1} TA stock solution was mixed with 200 µL of algal suspension. The TA concentration was based on pre-experimentation in order to inhibit but not kill the algae. The plates were shaken gently at 60 r.p.m. Daily and at the same time of day, chl fluorescence and photosynthetic yields were determined as described above. Experiments were run with 4 replicates with exponentially growing cultures and lasted for 3 days. Growth rates were calculated from day 0 – 3, and the photosynthetic yields were calculated each day. Several values are missing for days 0 and 3, due to technical problems with the photosynthetic yield measurements.

Growth rate and photosynthetic yield inhibition in co-existence experiments with *M. spicatum* (exp. 2)

In the second experiment, we compared the sensitivity of *P. duplex* strains to *M. spicatum* in more ecologically relevant, co-existence experiments (Körner and Nicklisch, 2002; Hilt et al., 2006; Hilt et al., 2012). Erlenmeyer flasks (500 mL) were filled with 450 mL of MIII medium and 3.75 ± 0.25 g (fresh-weight (FW)) apical parts of *M. spicatum* (15 cm long) or plastic plants as controls. The apical parts were harvested 4 days in advance and kept in tab water in order to prevent allelochemical or nutrient leaching from the wounds. *M. spicatum* originated from Lake Flakensee and was maintained in the lab with tab water under artificial light in plastic boxes, rooted in the sediment. At the day of the experiment, apical parts were rinsed carefully with distilled water to remove attached algae. Sterile sausage skin dialyse bags regenerated cellulose Wienie-Pak Skinless Sausage Casings (Devro Teepak, Scarborough, Ontario, Canada), with a molecular cutoff weight of 7000 (ca. 30 cm long), were pulled over 40 mL bottomless Schott-bottles, fixed with rubber clamps and filled with 50 mL of algal culture with a starting concentration of chl. F_0 = 15 µg L^{-1} (Phyto-PAM measurements). Experimental conditions were the same as algal maintaining conditions. Exp 2 was carried out with four replicates per treatment and with exponentially growing cultures for both the *Myriophyllum*-treatments and controls. Chl. F_0 and yields were measured daily, beginning at the same time of the day. The experiment lasted for 3 days and growth rates were calculated from day 0 – 3. Photosynthetic yields were calculated each day.

Statistical analyses

Because the data were not normally distributed and showed no homogeneity of variance (even after transformation of the raw data), non-parametric Mann-Whitney-U tests (MWU) were used to compare growth rates and photosynthetic yields between treatments and controls for each *P. duplex* strain.

Inhibition levels of the growth rates and the photosynthetic yields were calculated by

inhibition (%) = $(V_C - V_T)/V_C * 100$,

where V_C is the mean value of the controls, and V_T is the value obtained from the treatment. Subsequently, the means of the inhibition levels of the treatment replicates were calculated.

Kruskal-Wallis tests were used to test for differences in sensitivity levels between the strains using the % inhibition values.

Comparisons of inter-lake strain sensitivities were performed using the %-inhibition values and analyses of variance (ANOVA) with subsequent Tukey post-hoc tests, at the $p < 0.05$

significance level. Impacts of the strain origin on sensitivity were determined by Mann-Whitney-U tests, where % inhibition was pooled amongst all strains from the macrophyte-infested water vs. all strains from the macrophyte-free water. Correlations of sensitivity levels between both experiments and between yields and growth rates in the same experiments were made by Spearman rank correlations using % inhibition. A correlation between inhibition levels in % and control growth rates was performed by regression analysis. All statistical analyses were completed with the software package PASW 17 (SPSS).

Results

Species and strain selection

The ITS rDNA phylogeny showed that the *Pediastrum duplex* strains belong to no less than 8 lineages, numbered I-VIII, which presumably represent different species (Fig. 16A). Based on this phylogeny, the 23 strains from the most abundant *P. duplex* lineage (lineage V in Fig. 16A), containing strains from all four study ponds, were selected for subsequent experiments.

Strain-specific sensitivities to TA (exp. 1)

Growth rates of 10 out of the 23 *P. duplex* strains were significantly lower in the presence of TA, while the growth rate of one strain (Kalken49) was significantly increased by TA (Fig. 17). Between the strains, significant differences in sensitivities were observed, ranging from 24% (Kalken49) increase to a maximum of 58% (Kalken55) growth rate inhibition (Kruskal Wallis test, $p < 0.001$). Testing the effect of strain origin by comparing sensitivities of strains between the four lakes revealed two different sensitivity levels, with all strains from Kalken (mean inhibition 5%, data not shown) being least sensitive and Teufel (mean inhibition 29%, data not shown) being the most sensitive (one-way ANOVA with subsequent Tukey post-hoc tests, $F = 6.05$, $p < 0.001$, Fig. 17A). *Myriophyllum* presence in the pond or lake had no significant effect on strain sensitivities to TA (MWU test between macrophytes vs. no macrophytes, $p = 0.45$).

The photosynthetic yields mostly declined during the first 24 h in the TA treatments (exceptions: KL8, KLN10, Kalken56) and, to a lesser extent, in the controls (exceptions: Molen13, KL8, KLN10, Kalken56, Kalken59; Fig. 18). This resulted in significantly inhibited yields by TA addition for 14 strains at day 1 (Fig. 18, Table 12) and significant differences in sensitivities (Kruskal Wallis test, $p < 0.001$).

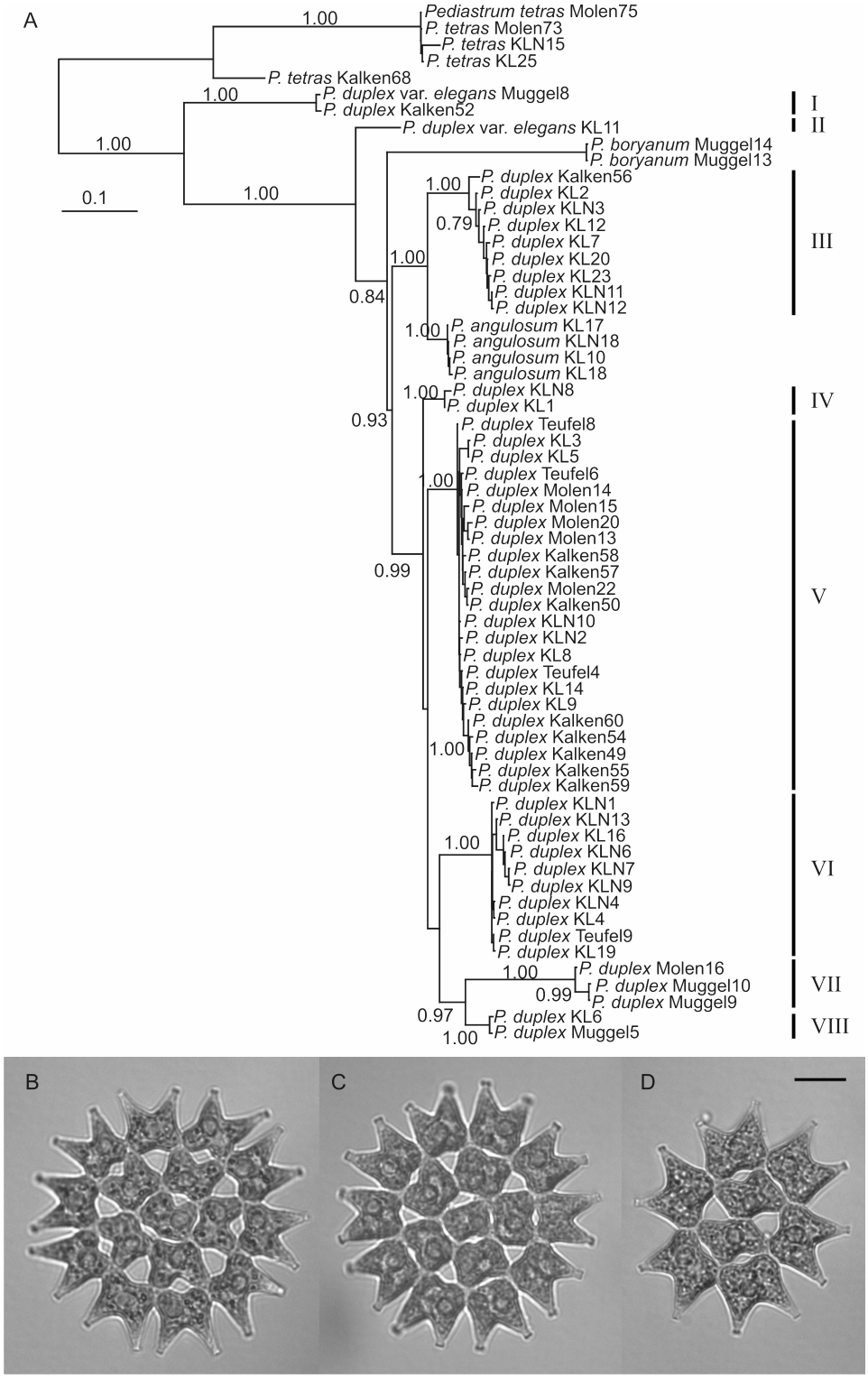

Figure 16:

ITS rDNA phylogeny of *Pediastrum* strains (A) and light microscopy photographs of three of the *Pediastrum duplex* strains from lineage V (B-D), used to study sensitivity to *Myriophyllum* allelochemicals. (A) Most likely phylogeny from a Bayesian Inference analysis. Posterior probabilities > 0.70 are shown at the respective nodes. The different *P. duplex* lineages recovered are numbered I-VIII. (B) *Pediastrum duplex* strain KL5, (C) Kalken49, (D) Molen20.

By day 2 and/or 3, most of the photosynthetic yields increased (Fig. 18). On day 2, 16 strains were significantly inhibited by TA and one strain was significantly enhanced (KL9) by TA (Table 12), showing significant different sensitivities (Kruskal Wallis test, $p < 0.001$). On day 3, the photosynthetic yields of 7 strains were significantly inhibited compared to the controls, and different sensitivities were again observed (Kruskal Wallis test, $p < 0.001$). The inhibition of the photosynthetic yield was not correlated to the presence of *Myriophyllum* at the strain origin (MWU-tests, $p = 0.52$, 0.61, 0.07 for day 1, 2 and 3, respectively). Due to technical problems, several measurements on day 0 and day 3 yielded defective data, which were excluded from the analyses (Fig. 18). A correlation between yield inhibition and growth rate inhibition by TA was found for day 3 (Spearman, $r = 0.27$, $p = 0.02$, but not for day 1 and 2 (Spearman, $r = 0.17$, $p = 0.11$ and $r = -0.08$, $p = 0.45$, respectively).

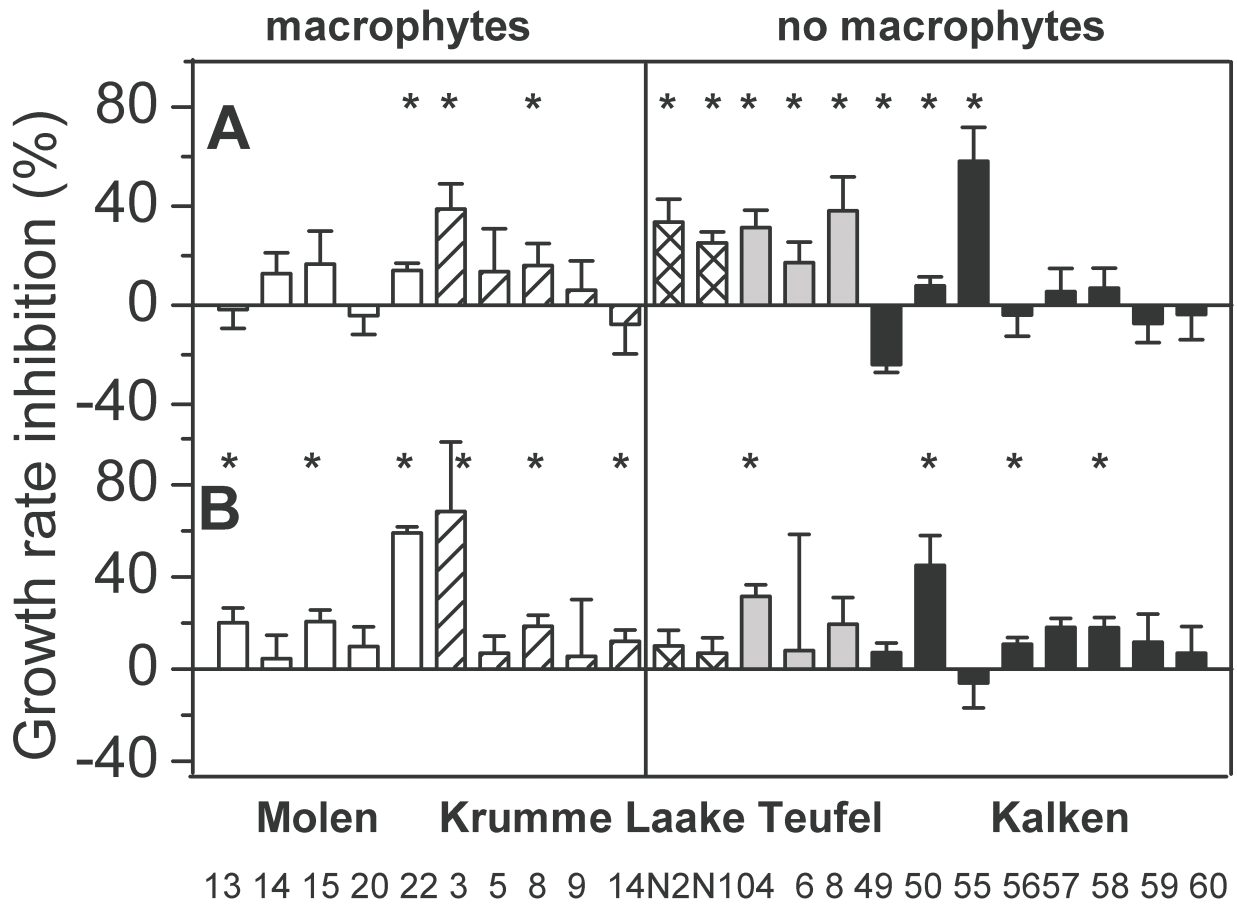

Figure 17:

Inhibition of the growth rates of 23 *Pediastrum duplex* strains isolated from lakes with and without allelopathically active macrophytes as compared to controls after 3 days of incubation with tannic acid (A) or in co-existence with *Myriophyllum spicatum* (B). Asterisks indicate a significant difference between the treatments and controls based on Mann-Whitney U tests. Error bars indicate the standard deviation. The strain numbers are given on the X-axis.

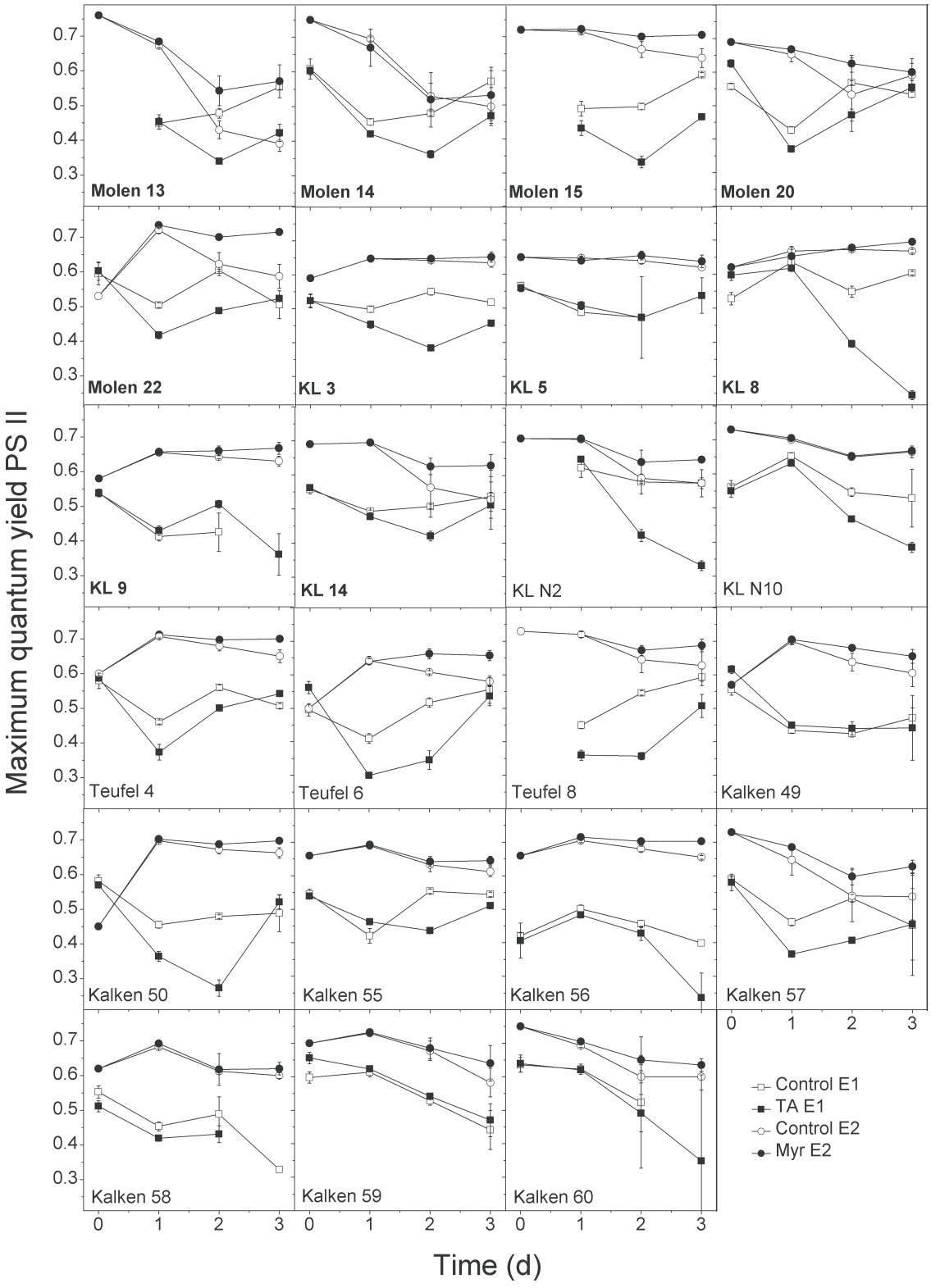

Figure 18:

Maximum quantum yields of photosystem II of 23 *Pediastrum duplex* strains isolated from lakes with and without allelopathically active macrophytes during 3 days of exposure to tannic acid (squares) or *Myriophyllum spicatum* (circles). Open symbols refer to the controls, and black symbols refer to the allelochemical treatments. Strains written in bold originated from macropyhte-dominated lakes. Error bars indicate the standard deviation.

Table 12: Results of Mann-Whitney-U tests (*p* values) comparing the maximum quantum yields of PS II between treatments and controls for the respective days and experiments. Significant differences between treatments and controls are indicated by bold letters. Dashes indicate that no test was possible due to invalid data obtained from the I-PAM. On day 0 of the *Myriophyllum* experiment, only the stock cultures were measured and the obtained value applied for all samples.

Lake		Strain no.	Tannic acid				*Myriophyllum spicatum*			
			Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
Molen	macrophytes	13	-	0.77	**0.02**	**0.02**	1	**0.03**	**0.02**	**0.02**
		14	0.24	**0.02**	**0.02**	**0.02**	1	0.75	1	0.47
		15	-	**0.02**	**0.02**	**0.02**	1	0.09	**0.02**	**0.02**
		20	**0.02**	**0.02**	**0.02**	**0.02**	1	0.16	**0.04**	1
		22	0.37	**0.02**	**0.02**	0.56	1	0.04	**0.02**	**0.02**
KL		3	0.77	**0.02**	**0.02**	0.16	1	0.87	0.19	0.10
		5	0.15	**0.04**	1	-	1	0.15	0.1	0.21
		8	0.08	0.07	**0.02**	**0.02**	1	0.41	0.19	**0.02**
		9	0.66	0.15	**0.02**	-	1	0.75	0.03	**0.02**
		14	0.39	**0.02**	**0.02**	0.48	1	0.74	0.14	**0.04**
KL	no	N2	-	0.08	**0.02**	**0.02**	1	0.62	0.08	**0.02**
	macrophytes	N10	0.25	**0.02**	**0.02**	0.06	1	0.13	0.65	0.74
Teufel		4	0.39	**0.02**	**0.02**	0.06	1	0.44	0.10	**0.02**
		6	**0.03**	**0.02**	**0.02**	0.39	1	0.88	**0.02**	**0.02**
		8	-	**0.03**	**0.02**	**0.02**	1	1	0.18	0.06
Kalken		49	**0.02**	**0.04**	0.25	1	1	0.34	**0.02**	0.06
		50	0.39	**0.02**	**0.02**	0.39	1	0.19	0.09	**0.02**
		55	0.25	**0.02**	**0.02**	**0.02**	1	0.65	0.37	**0.03**
		56	1	**0.03**	0.08	0.18	1	0.06	**0.03**	**0.02**
		57	0.77	**0.02**	**0.02**	0.77	1	**0.04**	0.25	0.06
		58	0.08	**0.02**	0.17	-	1	0.12	0.24	0.10
		59	**0.02**	0.47	0.08	0.56	1	0.65	0.65	0.19
		60	0.77	0.77	0.77	-	1	0.3	**0.02**	0.1

Strain-specific sensitivities to *Myriophyllum spicatum* (exp. 2)

Out of 23 tested strains, growth rates of 10 strains were significantly inhibited when cultured in co-existence with *M. spicatum* (MWU-tests, Fig. 17B). The strains had significant different sensitivities ranging from 6% enhancement (Kalken55) to 68% inhibition (KL3) (Kruskal Wallis test, $p < 0.001$, Fig. 17B). However, no differences in inter-lake strain inhibition levels were found (one-way ANOVA with subsequent Tukey post-hoc tests, $F = 0.67$, $p = 0.57$, Fig. 17B). The presence of *Myriophyllum* in the ponds did not affect strain sensitivity towards *Myriophyllum* (on average 23% and 15% inhibition, respectively, MWU-test, $p = 0.3$, data not shown). Moreover, the inhibition of *P. duplex* growth rates by TA and by *Myriophyllum* were not correlated (Spearman correlation, $r = 0.17$, $p = 0.1$).

The photosynthetic yields of *P. duplex* strains at day 0 varied, but consistently levelled off to values around 0.7 by day 1, with only slight differences (and only 2 were significant) between the controls and treatments (Fig. 18, Table 12). Thereafter, the photosynthetic yields of the controls slightly declined (exceptions KL3, KL8), whereas the treatments with *M. spicatum* either stayed constant or decreased less (Fig. 18). The photosynthetic yields of 9 strains were significantly greater by day 2 when in the presence of *M. spicatum* as compared to the controls (Table 12). The strains had significantly different sensitivities (Kruskal Wallis test, $p < 0.001$), with Molen13 being the most (26% higher) enhanced strain. By day 3, the yields of 12 strains were significantly greater in the *Myriophyllum* treatments when compared to the controls, showing significant different inhibition levels (Kruskal Wallis test, $p < 0.001$).

Photosynthetic yields of strains from ponds with macrophytes were not different from macrophyte-free water bodies (MWU tests, $p = 0.38$, 0.23 and 0.57 for day 1, 2 and 3, respectively). Enhancement levels of the yields and growth rate inhibition, due to *Myriophyllum* exposure, were not correlated (Spearman correlation, day 1: $r = -0.16$, $p = 0.13$; day 2: $r = -0.16$, $p = 0.14$; day 3: $r = -0.05$, $p = 0.61$). The inhibition/enhancement levels of the photosynthetic yields between both experiments were also not correlated (Spearman correlation, day 1: $r = -0.13$, $p = 0.24$; day 2: $r = 0.02$, $p = 0.84$; day 3: $r = -0.08$, $p = 0.5$).

Discussion

Strain-specific sensitivities towards allelochemicals

We reveal strain-specific differences in the sensitivity of *P. duplex* to polyphenolic allelochemicals when from the same ITS lineage. Between-strain variability is known for a number of physiological and biochemical characters (e.g., Fisher et al., 1973; Behra et al., 1999) and has consequences for allelopathic vulnerability as it constitutes an important

ecological trait of phytoplankton. Genetic diversity at the strain level has been underestimated in studies of species-specific phytoplankton responses to allelochemicals, but is important given the potential consequences of genetic diversity of primary producers on other ecosystem functions. Vanormelingen et al. (2009) showed strain-specific differences in grazing resistance traits in the green alga *Desmodesmus armatus*. The presence of sufficient intra-population genetic variation allowed local genetic adaptation to the grazing pressure in the respective habitats (Vanormelingen et al., 2009). Carillo et al. (2003) explained the high spatiotemporal heterogeneity of toxin production in natural populations of the cyanobacterium *Microcystis aeruginosa* by the detected high inter-strain variability of this trait. Even second-order effects of primary producers genetic diversity on higher trophic levels can be expected, such as those reported for seagrass strains by Reusch et al. (2005).

Our measured strain-specific sensitivities of *P. duplex* to allelochemicals differed depending on the used endpoint (growth rate versus photosynthetic yield), confirming the findings of Hilt et al. (2012) and Eigemann et al. (2013) (allelochemical sensitivities) and that of Foster (1982) (metal sensitivity of chlorophyta strains).

Additionally, the alga strain-specific sensitivities depend on type and concentration of the tested allelochemical and/or its method of addition. Differences in the response of organisms to different methods of allelochemical addition were previously emphasized by Reigosa et al. (1999).

Both single TA additions and continuous release of polyphenolic allelochemicals by *M. spicatum* inhibited the growth rates of about half of the tested strains (Fig. 17A, B). Nevertheless, only five strains experienced inhibition by both tested allelochemicals. These differences are not caused by changes in growth rates of strains during experimentation i.e., different physiological states (regressions between growth rates of controls and inhibition of treatments: exp. 1: $R^2 = <0.001$, $p = 0.89$, exp. 2: $R^2 = 0.006$, $p = 0.46$, data not shown). Different concentrations of atrazine (a herbizide inhibiting the electron transport from Q_A to Q_B similar to TA, Leu et al., 2002) caused different rankings of sensitivities of various *S. subspicatus* strains (Behra et al., 1999), and different TA concentrations resulted in different sensitivity rankings of three algal species (Eigemann et al., 2013). Assuming an excretion rate of 5 µg L^{-1} total phenolic compounds per day for *M. spicatum* biomass in exp. 1 (as measured for *M. verticillatum*, Hilt et al., 2006) and neglecting microbial and photolytical decomposition, allelochemical concentrations after 3 days would be two orders of magnitudes lower than TA concentrations used in exp. 1. Light availability might be another potential mechanism explaining different sensitivity rankings in both experiments (80 and 200 µmol

photons m^{-2} s^{-1} in exp. 1 and 2, respectively). Under low light conditions, the effect of the macrophyte *S. aloides* on the green alga *S. obliquus* was more pronounced than under high light conditions (Mulderij et al., 2005). In our study, however, the inhibition levels were the same between the experiments (t-test, $p = 0.08$).

Interestingly, the photosynthetic yields of PS II in numerous strains were significantly inhibited by TA, whereas a slight increase was observed in some strains in coexistence with *M. spicatum*, although an inhibition of PS II activity has been shown for *M. spicatum* (Körner and Nicklisch, 2002). Potential interfering effects on algal PS II yields include nutrient limitations (Parkhill et al., 2001; Nicklisch and Steinberg, 2009), which we eliminated by supplying nutrient-rich medium (Körner and Nicklisch, 2002), or shading effects (by the macrophytes and/or self-shading, Falkowsky, 1984). Importantly, lowered light intensities are known to increase photosynthetic yields (Genty et al., 1989). We used plastic plants in the controls to simulate shading effects by the macrophytes, but cannot fully exclude differences in light qualities and/or quantities between treatments and controls. Measurements of F_m that are lower than the actual F_m value are also possible when state transitions occur (Bonaventura and Myers, 1969), a phenomenon known for numerous cyanobacteria and a few green algal species (Campbell et al., 1998; Spijkerman, 2010). We tested whether state transitions occur for *P. duplex* by measuring PS II quantum yields in 3 strains (Molen14, KL14, Kalken60) with and without dark adaptation. Lowered F_m values after dark adaptation were not detected (data not shown), thus, state transitions in *P. duplex* seem unlikely to explain increased yields in the *Myriophyllum* treatments. Increased maximum PS II yields for algae after exposure to humic substances were also observed by Bährs and Steinberg (2012) and explained by interference with the electron transport chain. Further, Spijkerman (2010) found increased maximum quantum yields for the green alga *Chlamydomonas acidophila* by co-limitation for carbon dioxide and inorganic phosphorus. A conclusive explanation for this phenomenon, however, was not provided in any of these studies.

Correlation of sensitivities with the strain origin

Adaptations to unfavourable conditions are common in phytoplankton ecology (e.g., Fisher et al., 1973; Murphy and Belastock, 1980; Wood and Leatham, 1992). Depending on the exposure time to certain environmental conditions, physiological changes, epigenetic adaptations (gene regulation and/or gene expression), or genetically based adaptations can occur for phytoplankton populations (Lakeman et al., 2009). Contrary to our expectations, differences in sensitivities of *P. duplex* strains to allelochemicals were not correlated with the

presence of allelopathically active macrophytes in the lake of their origin as suggested by Al-Sheri (2010). Vanormelingen et al. (2009) were the first to detect local genetic adaptation for grazing resistance traits in natural populations of the green alga *D. armatus* that were exposed to contrasting grazing pressures by zooplankton whereas a fast genetic adaptation of the green alga *Dictyosphaerium chlorelloides* to moderate acidic and metal rich waters was found by López-Rodas et al. (2011). Rapid genetic adaptation of phytoplankton populations may be driven by the large genetic variation often found within populations (e.g., Rynearson and Armbrust, 2005; Vanormelingen et al., 2009) as well as fast growth and large population sizes, which would increase the chance that beneficial mutations appear. We could not confirm local genetic adaptation and the potential co-evolution between allelochemical donor and acceptors as suggested by Reigosa et al. (1999), Callaway and Aschehoug (2000) and Al-Sheri (2010). However, no information is available on the period of coexistence between *P. duplex* and the allelopathically active macrophytes in the sampled water bodies.

Algal cultures originating from different environments may adapt towards the culture conditions and loose specific traits (Lakeman et al., 2009). In addition, if population sizes are small or passing through a bottleneck that strengthens the selection, the genetic drift will be increased (Reed et al., 2003; Lakeman et al., 2009). These features are in principle given by isolation, maintenance and sub-culturing (Lakeman et al., 2009). However, our strains were maintained in the laboratory for a limited period of time, the cell numbers in the stock cultures were low, and for re-inoculation thousands of colonies were transferred. Consequently, the chances of such mutations can be considered to be low. Laboratory cultures are needed as they allow comparisons due to controlled conditions (Lakeman et al., 2009), which also decrease general stress (Reed et al., 2003). We furthermore used single cell isolation and brought all cultures to identical conditions in order to adopt the same selective pressure, which is supposed to be the best solution for comparing different strains of one algal species (Lakeman et al., 2009).

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Chapter VI:

Synthesis

Allelopathy is a complex process that has been widely used in terrestrial plant communities to explain patterns of dominance or changes in community structure that cannot be explained by either competitive or trophic interactions. It also occurs among all types of aquatic primary producers, but a full proof for the involvement of allelopathy in their interactions has still been difficult to establish (Gross et al., 2012). In this dissertation, I firstly focussed on the discovery of new observation variables in the phytoplankton that facilitate the separation of allelopathic effects from other interactions between aquatic macrophytes and the phytoplankton. I was able to find the inhibition of the esterase activity by polyphenolic allelochemicals as a new observation variable that allows an early and sensitive detection of a phytoplankton response, and thus potentially prevents interference with other processes during *in situ* measurements. Secondly, I investigated factors that potentially influence the sensitivity of the phytoplankton to allelochemicals, namely their species-specific bacterial colonization, interactions with other phytoplankton species and adaptation of strains to allelochemicals. Interactions between phytoplankton species were shown to significantly affect the sensitivities of species up to a reversal of the effects to allelochemicals. In contrast, a significant involvement of species-specific bacteria or strain-specific adaptation to allelochemicals could not be detected. I could furthermore show that the mode of allelochemical addition, allelochemical concentration, exposure time and the chosen observation variable influence the sensitivity of the phytoplankton to allelochemicals. In this chapter, I discuss the outcomes of my thesis chapters in a broader context and offer a preview on what could be the next steps in the research field of aquatic allelopathy.

VI.I. New observation variables for proving allelopathic effects in phytoplankton

Despite recent improvements in understanding allelopathic effects in aquatic environments (e.g., Gross et al., 2007; Mulderij et al., 2007; Hilt and Gross, 2008), it is still difficult to unravel allelopathy from effects caused by competition. Allelopathic effects on phytoplankton have indeed been shown for numerous aquatic macrophyte species (n = 37 in Mulderij, 2006), but mostly in laboratory experiments. A direct proof of allelopathic effects at the ecosystem scale has yet to be made. This lack of direct evidence is mainly caused by unsuitable

observation variables that fail to separate allelopathy from other interactions between macrophytes and phytoplankton, which leads to only few studies conducted under *in situ*-like conditions (e.g., Hilt et al., 2006). This problem was addressed in chapter II by searching for new methods and variables for the detection of allelopathic effects. The assessment of esterase activity inhibition by flow cytometry (chapter II) provides a new approach to tackle the difficulties of proving allelopathy *in situ*, compared to the known observation variables. The comparison between esterase activity (EA) and photosynthetic yield (PS) or cell division (CD) of the freshwater diatom *Stephanodiscus minutulus* (Fig. 19), illustrates the earlier and more sensitive detection of allelopathic effects (significant from 0.6 and 3 µmol L^{-1} tannic acid (TA) onwards compared to photosynthetic yield and cell division, respectively, Mann-Whitney-U tests, data not shown) by the use of esterase activity compared to the established variables.

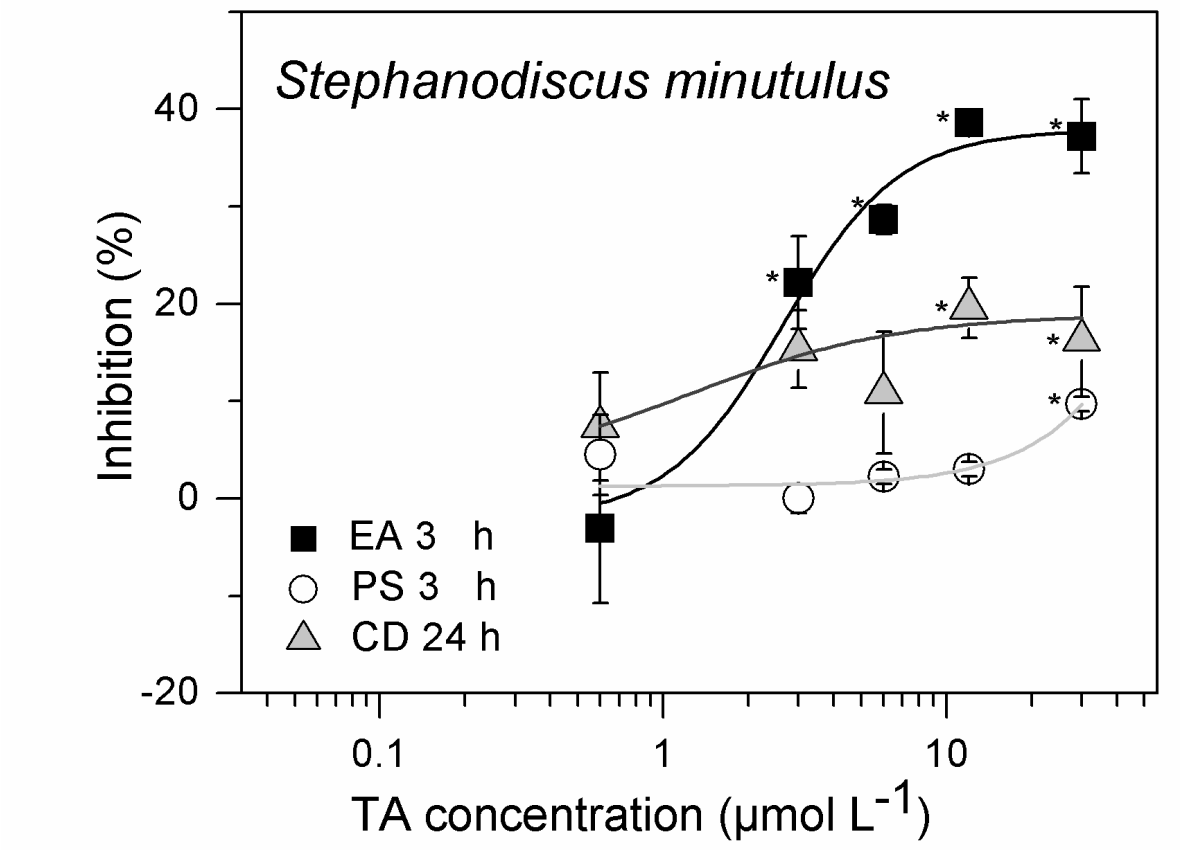

Figure 19:

Comparison of the inhibition of esterase activity (EA), the photosynthetic yield of the photosystem II (PS) and cell division (CD) of the diatom *Stephanodiscus minutulus* after exposure to TA. Asterisks indicate a significant difference ($p \leq 0.05$) compared to the controls. Error bars indicate the standard error (SE). Concentration–response curves are modelled with the log-logistic equation: $Y = A_2 + (A_1-A_2)/(1+(X/X_0)^p)$.

As a consequence, the inhibition of esterase activity as an observation variable passed the challenge of being useful for proving allelopathy *in situ* with respect to short exposure periods and naturally occurring allelochemical concentrations, two of the main constraints of the previously existing methods. Nonetheless, the activity of the esterase is potentially influenced by resource competition and nutrient limitation (Brookes et al., 2000a; b). These interferences, however, can be compensated by short exposure times. Algae possess internal nutrient stores that prevent effects on the esterase activity at least during the first hours of limitation or competition (Körner and Nicklisch, 2002). With small adaptations to the flow cytometric set-up, measurements of esterase activity by flow cytometry may be suitable for applications in co-existence experiments (which are close to *in situ* conditions), as conducted in chapters IV and V. However, my approach has as yet only been applied to single species-cultures and not to natural mixed phytoplankton communities. Consequently, its application for *in situ* studies of allelopathic effects at ecosystem level still requires additional investigation. Nevertheless, determination of the esterase activity of phytoplankton as a newly established observation variable is expected to accelerate insights into the relevance of allelopathic processes in aquatic ecosystems in forthcoming studies.

VI.II. Factors influencing the sensitivity of the phytoplankton to allelochemicals

The strength of allelopathic effects on the acceptor organism is modulated by a variety of parameters. A number of abiotic parameters have been shown to influence the sensitivity of the phytoplankton to allelochemicals (Bauer et al., 2012). However, also the target organisms themselves exhibit specific traits like differences in cell wall construction and uptake mechanisms or differences in the physiological target processes that may influence their sensitivities to allelochemicals (Hilt and Gross, 2008). Additional factors that were examined in this thesis are illustrated in Fig. 20 and discussed below.

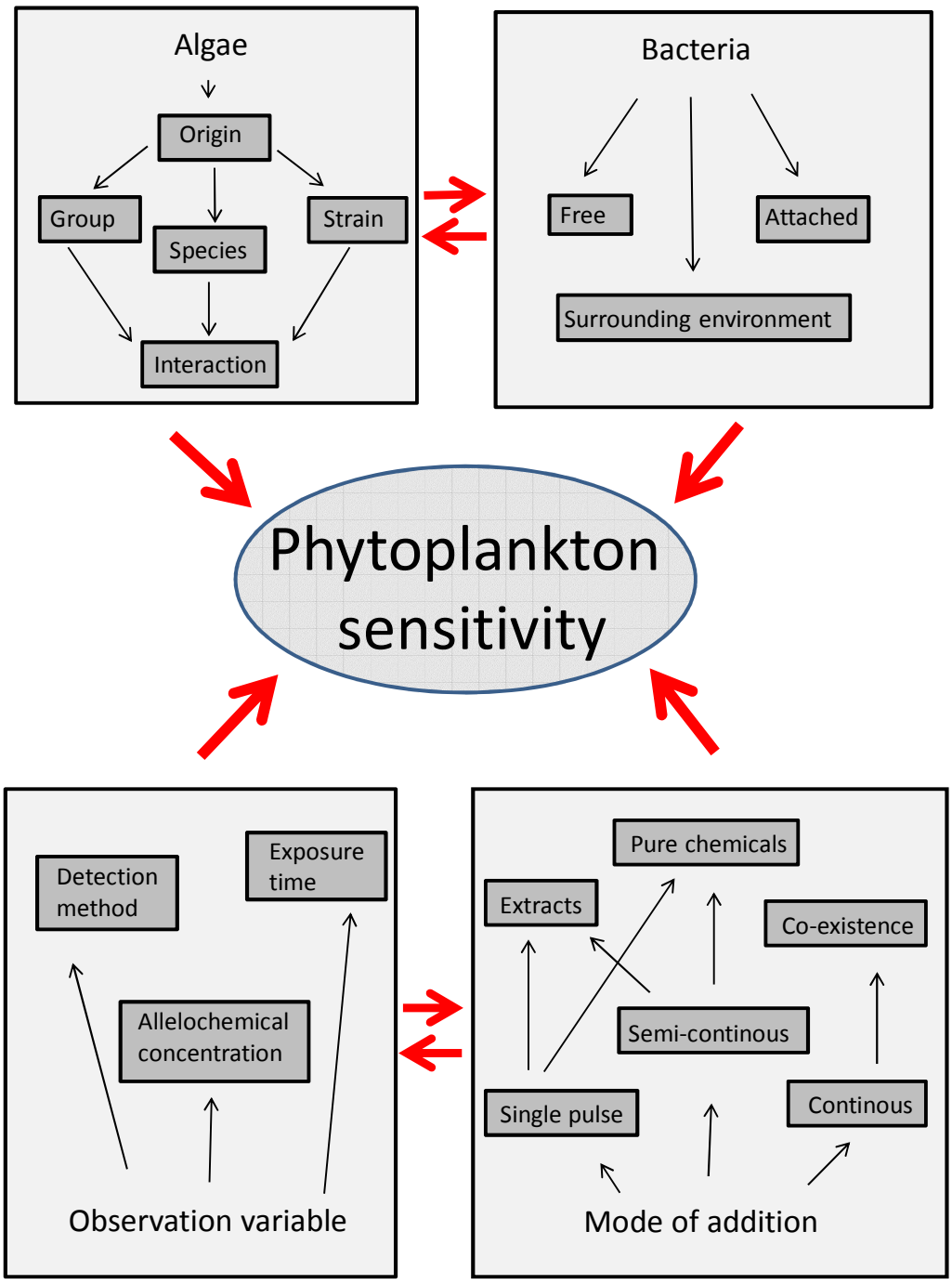

Figure 20:
Factors influencing the sensitivity of phytoplankton to macrophyte allelochemicals.

The origin and evolutionary history of the target algae are supposed to influence their sensitivity to allelochemicals (Reigosa et al., 1999; Al Sheri, 2010). Interestingly, local adaptation of the investigated strains of *Pediastrum duplex* to allelochemicals was not found (chapter V). However, adaptation processes can change sensitivities of algae (Fisher et al., 1973; Murphy and Belastock, 1980), and therefore the origin of an alga needs to be

considered (Fig. 20). Different algal groups exhibited different sensitivities to allelochemicals, and diatoms and cyanobacteria appeared to be more sensitive compared to chlorophytes (Hilt and Gross, 2008; Fig. 20). This finding was supported for most of the measurements in my work where single species cultures were used (chapters II, III and IV).

The same holds true for different phytoplankton species in one group (Fig. 20), exhibiting species-specific sensitivities to allelochemicals in the same order of magnitude as between groups (Jasser, 1995; Nakai et al., 1999; Körner and Nicklisch, 2002; Mulderij et al., 2005). Such species-specific sensitivities were confirmed in this thesis for the green algae *Desmodesmus armatus* (chapter II, III and IV), *Scenedesmus vacuolatus* (chapter II) and *P. duplex* (chapter V), the diatom *S. minutulus* (chapter II and III) and the cyanobacterium *Microcystis aeruginosa* (chapter IV). In addition to these known differences, I could furthermore show for 23 *P. duplex* strains that the sensitivity of phytoplankton to allelochemicals is also strain-specific (chapter V).

However, results of chapter IV revealed that single phytoplankton species tests, which are most common in aquatic allelopathy research (Hilt and Gross, 2008), do not necessarily reflect outcomes of allelopathic effects *in situ*, where diverse phytoplankton communities occur. Under natural conditions, interactions between different phytoplankton groups, species and strains will occur, which were shown to influence their sensitivities to allelochemicals (chapter IV; Fig. 20).

Another factor that modulates the sensitivities to allelochemicals is the association of bacteria with the target algae (chapter III; Bauer et al., 2010; De Figueiredo et al., 2011; Fig. 20) and bacteria in the surrounding environment (Müller et al., 2007). Hints of bacterial involvement into sensitivity differences were already detected by Casamatta and Wickstrom (2000), where differences in sensitivity between toxic and non-toxic strains of *M. aeruginosa* were attributed to their different microbes. My work (chapter III) showed that freshwater algal species harbor species-specific bacterial communities, which displays a prerequisite for the direct involvement of bacteria into different sensitivities. I was furthermore able to obtain several DNA sequences from known allelochemical degrading bacteria that were associated with the target algae (chapter III). I could not show, however, that a rather insensitive green alga harbors a higher share of potential allelochemical degrading bacteria than a sensitive diatom (chapter III). Still, it is clear that bacterial associations to the target algae are involved with their sensitivity to allelochemicals (chapter III; Bauer et al., 2009; 2012).

Different observation variables impacted the rankings of sensitivities between phytoplankton species (chapter II; Behra et al., 1999; Hilt et al., 2012) and strains (chapter V; Fig. 20).

Consequently, sensitivities of different phytoplankton groups, species and/or strains are difficult to compare when using different observation variables. From an ecological point of view, however, the growth rates of the targets are the most important trait, which makes a consistent appraisal possible. Recently, Hilt et al. (2012) showed that even different detection methods of the same observation variable affected the sensitivity rankings. Cell counts and chl *a* fluorescence, both variables for algal biomass, showed converse outcomes after the addition of TA to algal cultures (Hilt et al., 2012). Additionally, different concentrations of TA (chapter II) or allelochemical extracts (Elakovich and Wooten, 1995) resulted in different sensitivity rankings of the phytoplankton (Fig. 20). Given that allelochemical concentrations in macrophytes change with the season (Gross et al., 1996; Bauer et al. 2009), it can be expected that allelochemical concentrations in the water will be similarly variable (Gross, 2000). Thus, the sensitivities of the different target algae are likely to change over the season.
In my work, different exposure times at the same allelochemical concentration caused different rankings in sensitivities (chapter II; Fig. 20). Additionally, the mode of addition and the dosage form of the allelochemicals was also shown to influence the sensitivity ranking of the tested phytoplankton (chapter IV and V; Nakai et al., 1999; Reigosa et al., 1999; Hilt et al., 2012; Fig. 20). However, exposure time, dosage form and mode of addition are not important factors under natural conditions where a continuous release of allelochemicals by the macrophyte takes place, but again show the high complexity of allelopathic interactions.
Considering all the factors influencing the strength of allelopathic interactions together, it is unlikely that distinct generalized sensitivity rankings of phytoplankton groups, species or strains exist. This is emphasized in this thesis with various outcomes of sensitivities for algal groups, species and strains, observation variable, allelochemical concentration, exposure time and interactions between the targets.

VI.III. Allelopathic effects in aquatic environments with respect to space and time

Recently, Inderjit et al. (2011) stated for terrestrial ecosystems that the production, fate and effectiveness of allelopathic compounds in soils are influenced by environmental conditions and evolutionary history, generating a need for allelopathic interactions to be studied across spatial and temporal scales. This holds also true for aquatic ecosystems and in this paragraph, I discuss how allelopathic effects of macrophytes on phytoplankton meet these requirements. In my dissertation, I focussed on effects on phytoplankton as the target organism of allelopathic effects of aquatic macrophytes (VI.II), but most factors exhibit reciprocal traits,

as abiotic and biotic factors not only influence the acceptor but also the donor organism and thereby the quality and quantity of allelochemical production (Inderjit et al., 2011). As example, the production of allelochemicals in aquatic macrophytes is known to be influenced by abiotic parameters like changes in light and nitrogen availability (Gross, 2003). Furthermore, the genetic variability and associated heterotrophic bacteria were supposed to influence the allelopathic capacity of the donor organisms (De Figueiredo et al., 2011).

At low temporal and spatial scales (microns to centimeters, seconds to hours) the targets of the released allelochemicals are organisms living attached to the macrophytes (Fig. 21), comprising bacteria, epiphytes and herbivores. Bacteria attached to the aquatic macrophyte *Myriophyllum spicatum* were able to degrade allelochemicals and thus may effectively decrease the actual allelochemical concentration in the surrounding water (Müller et al., 2007). Epiphytes, living adjacent to the macrophyte are supposed to represent the biggest competitor of the macrophytes for light, the major limiting factor (Gross et al., 2003). Thus, one might expect them to be primarily targets of the plants defence efforts. Indeed, Wium-Anderson (1987) found almost epiphyte-free macrophytes exhibiting strong allelopathic capacities. Herbivory on macrophyte tissues might decrease the strength of the allelopathic potential (Gross et al., 2001), whereas on the other hand herbivores might also liberate allelochemicals (Inderjit et al., 2011). Furthermore, *M. spicatum* increased its phenolic content in response to herbivores (Lemoine et al., 2009). However, one should remember that allelochemicals not only exhibit allelopathic effects on the targets, but also perform other ecological services such as plant defence against herbivores, nutrient chelation and regulation of microorganisms (Haslam, 1988; Walenciak et al., 2002; Hilt and Gross, 2008; Inderjit et al., 2011). For all target organisms at this small scale, however, especially the specific mode of action may be important for the consequences of allelopathic effects (chapter II; Fig. 21). An effective inhibition of the PS II of competitive primary producers by macrophyte allelochemicals, as shown in several studies (Körner and Nicklisch, 2002; Leu et al., 2002; chapter II), for example, seems to be an appropriate mode in the competition for light (Gross, 1999). I was furthermore able to show that the esterase activity in the phytoplankton is inhibited shortly after allelochemical exposure, which may likewise exhibit significant disadvantages of primary producers living close to the donor macrophyte (chapter II).

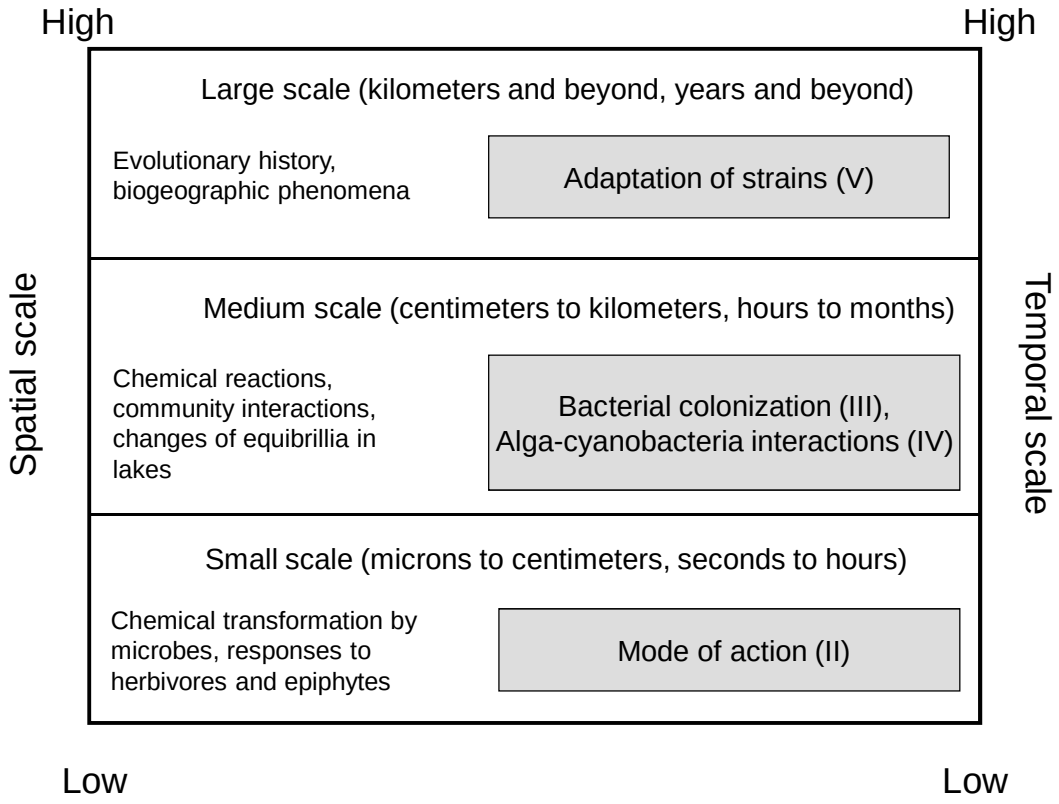

Figure 21:
Allelopathic effects in aquatic environments plotted against time and space, modified after Inderjit et al. (2011).

At the medium scale (centimeters to kilometers, hours to months), chemical reactions as well as organisms are assumed to shape allelopathic interactions (Inderjit et al., 2011; Fig. 21). Potential chemical processes in the aquatic environment are the degradation of exuded allelochemicals by oxidation, autolysis or UV-light cleaving which all depend on the environmental conditions (Müller et al., 2007; Bauer et al., 2012). Suspended and attached (to algae or particles) bacteria may utilize allelochemicals and thus rapidly decrease their active concentration (chapter III; Müller et al., 2007; Fig. 21), which may be compensated for by a continuous release (Nakai et al., 1999). In co-existence experiments, comprising macrophytes and the target phytoplankton, I could prove inhibition of the phytoplankton by macrophyte biomasses between 5 and 8 g L^{-1} wet weight, resembling natural conditions in allelopathically active macrophyte stands in shallow eutrophic lakes (chapters IV and V). The strength of allelopathic interactions however, differs between different target phytoplankton groups, species and strains, as shown in chapters II, III, IV and V. Consequently, allelopathy might be able to influence natural phytoplankton assemblages, which also has important implications for bacterial (chapter III; Bell and Mitchell, 1972) and zooplankton (Elser and Hassett, 1994)

community compositions. Another aspect at this scale is the interaction between different target organisms, which may also have significant impacts at the community level (chapter IV).

Considering all the impacts at this medium scale together, allelopathic effects may contribute to whole lake communities and ecosystems. Whole lake studies proposed allelopathy to be an important factor in the suppression of phytoplankton by macrophytes, and the frequent occurrence of allelopathically active macrophytes like *Myriophyllum*, *Ceratophyllum*, *Elodea* and *Najas* in temperate lakes suggested that allelopathy is an important mechanism in lakes dominated by the above-mentioned species (Hilt and Gross, 2008). As a consequence, allelopathic effects of macropyhtes on phytoplankton may support the clear water regime and prevent shifts to the turbid regime in eutrophic waters (Blindow et al., 2002; Lombardo, 2005).

On the large scale (kilometers and beyond, years and beyond), evolutionary aspects may also become important in the aquatic environment. The residence time of the allelochemicals may be influenced by the evolutionary history of the donor, because microorganisms that have undergone co-evolution with the allelochemical donor may use them as an energy source (Müller et al., 2007). Likewise, epiphytes on aquatic macrophytes (which face high allelochemical concentrations), were shown to exhibit lower sensitivities to allelochemicals than planktonic algae and cyanobacteria, also pointing to potential adaptation and co-evolution (Hilt, 2006). However, my studies did not confirm an adaptation of algal strains to allelochemicals (chapter V), but only local adaptation was tested.

As a consequence of adaptation and co-evolution, a biogeographic aspect of allelopathic interactions also arises (Reigosa et al., 1999; Inderjit et al., 2011). In the Novel Weapon Hypothesis (NWH, Callaway and Aschehoug, 2000), it was hypothesized that the success of certain invasive terrestrial plants can be attributed to the absence of co-evolution and adaptation of the acceptor species to the allelochemical donor, which may result in high sensitivities of native organisms to allelochemicals released by introduced plants. Similar findings have not yet been reported for the aquatic environment.

For an optimal evaluation of allelopathic effects, all possible scales of time and space should be considered. In my dissertation, I tried to approach all such scales in terms of time and space for allelopathic effects from aquatic macrophytes on the phytoplankton, starting from small scales (chapter II), over the medium scale (chapter III and IV) up to the large scale (chapter V; Fig. 21).

VI.IV. Outlook

Even though knowledge of allelopathic effects in aquatic environments has recently improved, it remains a superficially explored field of research. More investigations are needed to quantify factors that influence allelopathic interactions in aquatic environments at different temporal and spatial scales. A crucial question of allelopathic effects is to what extent co-evolution of donor and acceptor organisms has taken place (chapter V), and if allelopathy is a stable evolutionary trait at all (Gross et al., 2012). Attempts to explore possible co-evolution and adaptation of allelochemical donor and acceptor have been undertaken for terrestrial environments (Inderjit et al., 2011), but for the most part are lacking in aquatic environments (Gross et al., 2012). For an adequate evaluation of allelopathic effects, a combination of experimental approaches is required that involves potential impacts of all abiotic (e.g., light and nutrient interference) and biotic (e.g., grazing) factors. For the analysis of such attempts, sophisticated molecular methods may enable specific tests for the impacts of allelopathy on the metabolic level. Here, the biggest challenge will be the linkage of these results to ecologically relevant functions (Gross et al., 2012). Future studies should furthermore aim to identify more allelopathically active substances released from aquatic primary producers (Nam et al., 2008; Zhu et al., 2010; Gross et al., 2012), even though the strongest effect might be expected from the released mixture (Nakai et al., 2000). Identified allelochemicals allow the examination of their specific modes of action (chapter II; Zhu et al., 2010), and may thus lead to a better evaluation of their *in situ* impacts. Furthermore, sufficient data on release and active concentrations of allelochemicals *in situ* need to be determined with improved and more accurate methods (Nam et al., 2008), which would allow better appraisals of their actual effect. As a next step, the gained knowledge may be applied in two different directions of research. Firstly, *in situ*-like experiments (e.g., large scale outdoor co-existence experiments) that resemble natural conditions, and secondly, the establishment of accurate models for allelopathic effects of submerged macrophytes on phytoplankton based on detailed knowledge of the effects of numerous factors such as target strains and species (chapter II and V), bacterial mediation (chapter III) and interactions between acceptor organisms (chapter IV). Both directions may then contribute to a better understanding of allelopathic effects in aquatic environments.

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Statement of academic integrity

I hereby certify that the submitted thesis "Allelopathic effects of submerged macrophytes on phytoplankton: determining the factors of phytoplankton sensitivity and detection of new modes of action" is my own work, and that all published or other sources of material consulted in its preparation have been indicated. Where any collaboration has taken place with other researchers, I have clearly stated my own personal share in the investigation (see Outline of the thesis). I confirm that this work, in the same or a similar form, has not been submitted to any other university or examining body for a comparable academic award.

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Falk Eigemann

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