Mixotrophy and pelagic ecosystem dynamics

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MIXOTROPHY AND PELAGIC ECOSYSTEM DYNAMICS

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SUMMARY

Protist species were traditionally classified morphologically as either „plants“ or „animals“, based on the absence or presence of chloroplasts. State of science is that a high number of protist species carry chloroplasts but are nutritionally able to employ both autotrophy (photosynthesis) and heterotrophy within a single cell. This combination of autotrophic and heterotrophic mode of nutrition within a single species is named mixotrophy. In protists, heterotrophy can be realized either by the uptake of food particles (e.g. bacterial prey) through phagocytosis or by the uptake of dissolved organic compounds (i.e. osmotrophy).

Mixotrophy is globally and increasingly described in protists from all types of aquatic habitats. Plankton ecologists nowadays assess mixotrophy among the traditionally typified “phyto”plankton and mikro”zoo”plankton species as regularity. Nevertheless, detection and quantification of mixotrophy is still a methodological challenge. In this study, we focused on mixotrophy in marine phytoplankton species and put emphasis on its phagotrophic nutrition from heterotrophic bacterial prey.

State of the art methodology was tested to visualize mixotrophy in single phytoplankton cells. Catalyzed reported deposition-fluorescence in situ hybridization (Card-FISH), using 16S ribosomal RNA probes, was employed based on existing protocols for bacteria and protists. The method proved to be a valuable tool to visualise bacterial phylogenetic groups in association with phytoplankton by epifluorescence microscopy without need for prior isolation of cells or interference with the microbial association. However, the method failed to visualize mixotrophy in phytoplankton since the general eubacterial probe (EUB338) hybridised a broad range of phytoplankton species making it impossible to discriminate fluorescent signals originating from bacterial or phytoplankton tissue. The method showed additional weaknesses in its application to study mixotrophy in protists, particularly due to unavoidable and size-specific losses of bacterial and protist cells during its incubation steps (Chapter 1).

Two extensive laboratory experiments were realized to study influences of environmental factors on ecological phytoplankton-bacteria interactions, using the mixotrophic crysophyte *Ochromonas minima* as a model species (chapters 2 and 3). Background of these studies is phytoplankton and heterotrophic bacteria being major competitors for dissolved inorganic nutrients. In case that bacterial growth is carbon limited, increasing concentrations of degradable dissolved organic carbon (DOC) enhance bacterial growth and consumption of dissolved nutrients and thereby negatively affect autotrophic phytoplankton growth. Bacteria consuming mixotrophic phytoflagellates, however, may gain in importance in such situations since DOC provokes higher bacterial prey supply.

In the first laboratory growth experiment it was shown, in particular, that concentration and substrate type of DOC interactively influenced *O. minima* maximum abundance. The mixotrophic phytoflagellate generally profited from moderately augmented DOC concentrations. With increasingly higher DOC concentrations, the ultimate effect on *O. minima* abundance depended on the type of organic substrate added, and the potential of the carbon substrate to sustain continued growth of bacterial prey by importing nutrients (nitrogen, phosphorus).

The second laboratory growth experiment concentrated on the role of temperature and a single DOC source (saccharose) for the performance of *O. minima* in an artificially established phytoplankton community comprised of a total of six species. This study confirmed the positive influence of DOC on *O. minima* growth for situations in which the mixotroph grows under competition for nutrients with other phytoplankton species. DOC at highest experimental supply shifted phytoplankton community composition to the dominance of the mixotrophic model species. In addition, our results indicate a potential positive effect of temperature on *O. minima´s* heterotrophic nutrition mode, and indicate a potential increasing contribution of mixotrophic species to phytoplankton communities under increasing sea surface water temperatures.

RESUME

Les espèces protistes ont été traditionnellement classifiées comme des plantes or des animaux en raison de l'absence ou présence des chloroplastes. Cependant, l'état actuel de la connaissance indique qu'un grand nombre d'espèces protistes portent des chloroplastes mais que physiologiquement elles sont capables d'utiliser l'autotrophie (photosynthèse) ou l'hétérotrophie pour se nourrir. La combinaison de ces deux modes trophiques par un même organisme est nommée mixotrophie. Chez les protistes l'hétérotrophie peut s'effectuer soit par la consommation des particules par phagocytose, e.g. des proies bactériennes, ou bien par l'absorption des composants organiques dissouts, i.e. osmotrophie.
La mixotrophie est de plus en plus décrit chez les protistes dans tous les habitats aquatiques. Les écologistes du plancton constatent la récurrence de la mixotrophie chez les formes traditionnelles «phyto»plancton et micro »zoo»plancton. Cependant, identifier et quantifier la mixotrophie reste toujours un défie méthodologique. Dans cette étude nous nous sommes intéressés à la mixotrophie chez les espèces phytoplanctoniques marines, en particulier à leur nutrition phagotrophique de proies bacteriennes.

Afin d'identifier le caractère mixotrophe chez les cellules phytoplanctoniques, nous avons utilisés des techniques modernes de cytogénétique.. La technique cytogénétique d'hybridation in situ Card-FISH en utilisant de sondes d'ARN ribosomique 16S a été effectuée suivant des protocoles existant pour des bactéries et des protistes. Cette technique s'est avérée être un outil précieux pour visualiser des groupes phylogénétiques bactériens en association avec le phytoplancton à l'aide de la microscopie à épifluorescence, sans avoir besoin d'un isolement préalable des cellules ou des interférences avec l'association microbienne. Cependant, la méthode a échoué pour visualiser mixotrophie chez le phytoplancton car la sonde eubactérienne générale (EUB338) combine une large gamme d'espèces phytoplanctoniques, ce qui rend impossible de discriminer les signaux fluorescents provenant de tissus bactérienne ou phytoplanctonique. De plus, la méthode a montré des faiblesses supplémentaires dans son application à l'étude de la mixotrophie chez les protistes, notamment en ce qui concerne l'incontournables perte spécifique de certaines tailles des cellules bacteriennes et protistes pendant leur processus d'incubation (Chapitre 1).

Deux expériences approfondies ont été réalisées pour étudier l'influence des facteurs environnementaux sur les interactions écologiques phytoplancton-bactéries, pour ceci nous avons utilisé l'espèce mixotrophe crysophyte *Ochromonas minima* comme modèle (chapitres 2 et 3).
Le contexte écologique de ces expériences s'appuie sur la compétition pour des nutriments inorganiques dissouts entre les phytoplancton et les bactéries hétérotrophes. Dans le cas où la croissance bactérienne est limitée par le carbone, l'augmentation de la concentration de carbone organique dissous (DOC) renforce la croissance bactérienne et la consommation de nutriments dissous et ainsi affecte négativement la croissance du phytoplancton autotrophe. Cependant, les consommateurs de bactéries, i.e. phytoflagellés mixotrophes, peuvent etre favorisés dans de telles situations car la hausse de DOC donne lieu à l'abondance plus élevé des proies bactériennes.

Dans la première expérience de croissance, il a été démontré que la concentration et le type de substrat du DOC interagissent affectant l'abondance maximale *d'O. minima*. Le phytoflagellé mixotrophe est en général favorisé par la concentration légèrement accentuée de DOC. En situation des concentrations de DOC de plus en plus élevés, l'effet ultime sur l'abondance d'*O. minima* dépend du type de substrat organique ajouté et de leur potentiel de soutenir une croissance continue des proies bactériennes par la comsommation de nutriments (azote, phosphore).

La deuxième expérience de croissance a ciblé le rôle de la température et d'une seule source de DOC (saccharose) et leurs effets sur la performance de O. minima au sein d'une communauté phytoplanctonique créé artificiellement et composée d'un total de six espèces. Cette étude a confirmée l'influence positive du DOC sur la croissance de O. minima dans des situations où l'espèce pousse sous compétition pour les nutriments avec d'autres espèces de phytoplancton. Le plus grand apport de DOC a fait basculer la composition de la communauté phytoplanctonique vers la domination de O.minima. En outre, nos résultats indiquent un potentiel effet positif de la température sur le mode de nutrition hétérotrophe de l'espèce, ainsi qu'une croissante contribution des espèces mixotrophes au sein des communautés de phytoplancton dans des conditions des hautes températures des eaux de surface de la mer.

INTRODUCTION

Mixotrophy – A nutritional behaviour strategy

The general topic of this study is mixotrophy in marine phytoplankton. While traditionally, protists were morphologically classified as either “plants” or “animals”, based on the presence or absence of chloroplasts, state of the science realizes that protist species may either be purely autotrophic photosynthesizers, purely heterotrophic protozoa or mixotrophic species. Mixotrophy is the combination of autotrophic and heterotrophic nutritional forms within a single species. Protists can apply heterotrophic nutrition either by uptake of particulate organic carbon, such as bacterial prey particles, by phagocytosis and/or by the uptake of dissolved organic carbon (osmotrophy).

Mixotrophic protists are globally common in all types of aquatic habitats, reaching from oligotrophic freshwater lakes, to eutrophic coastal sights and open ocean areas (Burkholder et al. 2008, Sanders and Gast 2011, Zubkov and Tarran 2008). While many protist species are inheritably able to physiologically apply phototrophy and heterotrophy within the same cell, some ciliates and heterotrophic dinoflagellates have to take up chloroplasts from ingested prey to use these acquired and so-called “kleptoplastids” for their own cellular energy production (Stoecker 1999). Mixotrophy is an evolutionary strategy with a broad diversity of behavioural forms, reaching from almost pure autotrophy to almost pure heterotrophy (Jones 1997). The degree of activity of the relative nutritional modes is regulated by environmental factors and cell internal mechanisms and is species specific (Granéli et al. 1999). Mixotrophy provides the protists with ecological advantages in situations when environmental factors are infavourable for pure phototrophic or pure phagotrophic nutritional modes. Mixotrophic flagellates may employ heterotrophy to substitute photosynthetic carbon and energy supply if light conditions are limiting, whereas uptake of bacterial prey can be an important way to acquire nutrients and trace elements, if these are limiting in the inorganic dissolved pool (Arenovski et al. 1995, Gliber et al. 2012, Maranger et al. 1998). On the other hand, flagellates that usually tend to rely rather on phagotrophy, may switch on photosynthesis, if prey items are scarce.

This study presented here, concentrates on forms of mixotrophy, in which bacteria are taken up as prey by photosynthetic phytoflagellates.

Mixotrophy in the plankton – existence and detection

Mixotrophy among flagellates is increasingly reported in aquatic studies and planktologists now, assess mixotrophy in planktonic protists rather a rule than a special exception. Nevertheless, most studies that deal with mixotrophy focus on descriptions of occurrences of mixotrophs in diverse habitats (Arenovski et al. 1995, Nygaard and Tobiesen 1993) or on single species feeding behaviour (Stibor and Sommer 2003, Rothhaupt 1997), but only relatively few studies focuse on the role that mixotrophic phytoflagellates play for food web functioning.

One of the reasons for this discrepancy is surely the broadness of the mixotrophic strategy and its dependence on environmental factors (Jones 1997), which prevents a complete determination of the actual total abundance of mixotrophs in a certain environment. For instance, sampling surface water plankton for determination of abundance of mixotrophs may yield completely different results on sampling days with sunshine and clear sky, than during rainy and cloudy weather conditions, since, as described above, mixotrophs can down- or up-regulate the degree of phagotrophy with fluctuating light availability and the respective behaviour is dependent on the individual species.

In addition, in-situ authentification of mixotrophy is, in general still difficult and a methodological challenge. So far, for detection of mixotrophs, environmental or laboratory samples with potentially mixotrophic species were mainly incubated with artificial or dead natural fluorescent prey particles, to afterwards identify mixotrophs, based on epifluorescence microscopy, as chloroplast-bearing cells that had taken up fluorescent particles (Sherr and Sherr 1993). The general problem of these methods is that certain phytoplankton species may ignore artificially added tracer prey particles, since these may not match sufficiently the prey scheme. In addition, the preferred prey size for feeding may be different within phytoplankton species and therefore not all mixotrophs in an environmental samples may be found with a certain prey imitate. Moreover, the feeding activity of mixotrophic species follows functional responses on prey concentration (Hansen and Nielsen 1997) and therefore, an addition of artificial prey, may enhance prey concentrations in a way that increases the feeding activity of the protists. In the latter case, the method would eventually overestimate the natural importance of mixotrophic activity in a sample. However, the technical advance in molecular biology, especially the development of fluorescence in situ hybridisation (FISH), makes it possible to stain phylogenetic groups of bacteria directly in environmental samples. In this method, ribosomal RNA targeting oligonucleotide probes that are labelled by fluorescence dyes, enter fixed and permeabilized bacterial cells, hybridize its nucleic acid and thus label the cell for fluorescence microscopic or flow cytometric analysis (Amann et al. 1990a, Amann et al. 1990b). A limited number of newer studies have described FISH as a useful tool to study bacteria inside protists (Diederichs et al. 2003, Jezbera et al. 2005).

Ecosystem ecological background of this study

Even though studying mixotrophy is a technical challenge until now, studies already started to point out the potential crucial importance of this nutritional strategy for whole pelagic ecosystem functioning. For example, humic (and therefore DOC rich) lakes have often been described to be dominated by mixotrophic species because of the high availabilities of bacterial prey but the low availability of light for photosynthesis (Jansson et al. 1996, Jones 2000). Terrigenous dissolved organic carbon (tDOC), such as humic substances, naturally does not only influence freshwater habitats, but is washed out of soils and finally also transported by rivers to estuaries and coastal marine ecosystems. Under global change scenarios, it is predicted that precipitation is going to increase in the northern hemisphere, while at the same time melting permafrost soils are going to deliberate more DOC to be washed out (Freeman et al. 2001, Wickland et al. 2012). Northern coastal ecosystems, such as the northern Baltic Sea, are therefore going to be affected not only by increasing nutrient concentrations and warmer surface water temperatures, but also by increasing loads of tDOC.

Heterotrophic bacteria and autotrophic phytoplankton are the major competitiors for dissolved inorganic nutrients in aquatic habitats. Heterotrophic bacterial growth can be limited by inorganic nutrients and/or DOC. Under nutrient replete conditions, increasing concentrations of DOC thus provoke higher bacterial growth in carbon limited bacteria. In such situations, autotrophic phytoplankton usually declines, since increasing amounts of dissolved nutrients are consumed by bacteria, which compete better than phytoplankton for dissolved inorganic nutrients (Joint et al. 2002, Kirchman 2008, Thingstad et al. 2008). However, following a completely different nutritional strategy, mixotrophic phytoflagellates may behave differently than pure autotrophs – an assumption that is taken up by the study goals.

Goals of this study

This study had two major goals:

The first goal was to test whether it is possible to detect mixotrophy *in situ*, using the modern technique CARD FISH (catalyzed reporter deposition fluorescence in situ hybridization, chapter 1).

The second study aim was to elucidate the role of the environmental factors DOC and temperature for phagotrophic phytoflagellate performance, when grown under competition with heterotrophic bacteria.

For the latter, it was hypothesized, that if heterotrophic bacteria are nutrient-replete, but limited by carbon, increasing concentrations of DOC should provoke increasing abundances of mixotrophic phytoflagellates, since these are competitors but at the same time consumers of bacteria and should therefore profit from higher prey supply (chapters 2 and 3).
In this context, it was tested, whether warmer water temperature is an additional factor that positively influences success of mixotrophic phytoflagellates (chapter 3).

A further and connected study focus was the role of DOC on nutrient limitation of a mixotrophic flagellate. Jansson et al. (1996) have proposed mixotrophs in humic lakes to be generally limited by nitrogen (N), because of high uptake of phosphorous (P) by grazing on phosphorous-rich bacteria. In contrast, the study presented here, hypothesized that the generally high variability of N:P ratios of heterotrophic bacterial biomass should be reflected in the nutrient limitation patterns of the mixotrophs that prey upon heterotrophic bacterial communities, which are grown under different DOC supplies (chapter 2).

To achieve the study aims, CARD FISH was tested on laboratory cultures of non-axenic phytoplankton cultures and on natural marine seston samples. A possible improvement of the methodology was discussed.

To study effects of DOC and temperature on mixotrophic phytoplankton performance, two controlled laboratory growth experiments were established, using the mixotrophic chrysophyte *Ochromonas minima* as a model species.

O minima was first grown associated with its heterotrophic bacteria in gradients of three different DOC sources that differed from each other in their respective nutrient content (N, P). In a second experiment, *O. minima* was grown in monocultures and as part of an artificial laboratory community under a gradient of a simple, nutrient-free DOC source, at two different incubation temperatures.

CHAPTER 1

CARD-FISH AS A MOLECULAR METHOD TO VISUALIZE PHYTOPLANKTON ASSOCIATED BACTERIA - IS IT APPROPRIATE FOR DETECTION AND QUANTIFICATION OF MIXOTROPHY?

Vera de Schryver, Miguel Angel Ballen Segura, Kevin Nahélou

1.) Background: Traditional and modern methods for the visualization of colourless microbes and for the detection of mixotrophy

1.1 Visualization of microbes

While phytoplankton can easily be visualized by light microscopy due to its natural pigmentation of the chloroplasts, heterotrophic bacteria and heterotrophic nanoflagellates (HNFs) are non-pigmented and need to be stained artificially prior to microscopic detection, to achieve satisfying pictures.

The development of fluorescent dyes and epifluorescence microscopy was a revolution for microbiological studies. The first fluorescent dyes that were, and still are, used routinely for bacterial enumerations are so called nucleid acid stains; chemical substances such as acridine orange (3,6-tetramethyl diaminoacridine; Hobbie et al. 1977), DAPI (4´6-diamidino-2-phenylindole, Porter and Feig 1980), or the newer SYBR green (Patel et al. 2007), that bind easily and specific to any nucleic acid molecules present in pro- and eukaryotic cells. While originally, colourless aquatic microbes were fixed and filtered on membranes that could be stained and mounted on slides for microscopic counting and morphological studies, now, counting of stained cells from liquid samples by flow cytometry is also a routinely applied method in microbial biology.

State of the art molecular methods allow the direct visualisation and detection of whole cells of specific phylogenetic microbial target groups in mixed environmental microbial samples, without prior need for isolation and cultivation of the groups of interest. Formaldehyde, which is used for fixation of microbial cells, also makes the cells permeable for designed and synthesized oligonucleotide probes that target group- or species-specific regions of the ribosomal RNA (rRNA) for hybridization (Giovanni et al. 1988). Synthetic coupling of such specific oligonucleotide probes with fluorescent substances provides a powerful tool for sensitive and specific colouration of single target cells for epifluorescence microscopy and flow cytometry (Amann et al. 1990a, Amann et al. 1990b) and is known as “fluorescence in situ hybridization” (FISH).

A further development of this method is “catalyzed reporter deposition FISH” (CARD FISH), which is also called “tyramide signal amplification FISH” (TSA FISH), since it increases the fluorescence signal intensity up to 20-fold. In CARD FISH, horseradish-peroxidase (HRP) conjugated oligonucleotides are, in a first step, used for hybridization of target sequences. In a further working step, fluorescently labelled tyramide (as the reporter molecule) is incubated with the sample and HRP catalyzes its deposition in the target cell, which finally is intensively bright labeled (Schönhuber et al. 1997, Pernthaler 2002).

1.2 Detection of mixotrophy

The feeding process of phytoflagellates can generally be observed by light microscopy if the organisms prey on visible cells, such as other phytoplankton species or metazoans (Berge et al. 2012). It was also reported that mixotrophy in dinoflagellates can be concluded from orange fluorescent inclusions in the cell that originate from algal prey (Stoecker et al. 1997). However, if one aims to visualize feeding on heterotrophic bacteria by phytoplankton, labelling of artificial or dead natural prey with fluorescent dyes (Sherr and Sherr 1993) or radioactive substances (Stibor and Sommer 2003) has been unavoidable until recently. While these methods are well established, and allow to measure grazing rates, the interpretation of results may sometimes be questionable. This is because an addition of labeled bacterial prey into a natural or laboratory sample can augment prey concentrations so extensively that feeding rates of the phytoflagellates are influenced, since mixotrophic phytoplankton species´ feeding behaviour follows functional responses to prey concentrations (Hansen and Nielsen 1997). FISH provides a tool to visualize bacterial cells in environmental samples and was described as a successful method to detect feeding of ciliates and heterotrophic nanoflagellates by visualizing bacteria enclosed in food vacuoles (Diederichs et al., 2003, Jezbera et al., 2005). In our study, we tested a new CARD FISH method for the visualisation of phagotrophy in mixotrophic protists which was developed by Medina-Sánchez et al., 2005.

2.) Experiences with the Card FISH method in the study of mixotrophy

2.1 Loss of cells during application of CARD FISH

For applying the CARD FISH protocole described by Medina-Sánchez et al. 2005, phytoplankton samples were fixed and permeabilized with 2% formaldehyde (final concentration) and subsequently filtered on polycarbonate membranes. To avoid loss of cells during incubation, the membranes with the samples got embedded into agarose prior to incubations for fluorescence in situ hybridization. Nevertheless, we observed very high loss rates for phytoplankton cells during the protocol incubations when we used centrifuge tubes and similar laboratory vessels for incubating membranes (all at the same time) in reagent solutions. When applying the protocol to dinoflagellate species, we extended the protocole by an additional step in order to enzymatically permeabilize the dinoflagellates theca (Palacios and Marín 2008) and observed losses from the membranes of up to 100% of initially sampled cells.

To counteract this problem successfully, we highered the agarose concentration up to 0.4 %. Even higher agarose concentrations further improved the amount of sample that remained on the membranes throughout the protocole but caused high background fluorescence and therefore made microscopic analysis impossible.

However, to diminish mean loss rates throughout the protocole to approx. 30 % for a Rhodomonas sp. and to about 60 % for Prorocentrum micans, we had to replace our initially used incubation vessels. To achieve the latter results, we sticked to the original protocole and supplemented it by the step described in Palacios and Marín, 2008, but used parafilm-coated microscopy slides for incubating samples. Filters were carefully placed face-up on the slides and carefully covered with about 0.5 ml of incubation solutions. We did all incubation steps of membrane-samples on glass-slides in lid-covered plastic boxes in an temperature controlled culture incubator and hybridization oven, respectively. Washing steps were performed by carefully dropping washing solutions on the filter membranes (still places on glass slides) or by carefully drowning the membranes into small amounts of washing solutions in petri dishes. For the latter, filters were treated one-by-one to avoid contact between the samples.

To test the success of our changes to the protocole, we did microscopic countings on membranes covered with the same amounts of mixed samples of *Prorocentrum micans* and *Rhodomonas sp.*. Four Membranes were mounted on glass slides and counted on an epifluorescence microscope directly after filtering the sample, and to four other membranes the extended and improved protocole was applied prior to counting. Membrane specific loss rates of species were calculated as the proportion of cells per eyefield that remained after the protocole, relative to the mean of cells/per eyefield obtained from membranes that did not undergo the protocole. The procedure was repeated six times for *Prorocentrum micans* and three times for *Rhodomonas sp.* Pooling of the data reavealed a significantly, approximately two-times higher (ca. 60%) specific loss rate for *Prorocentrum micans* than for *Rhodomonas sp* ($t = 5.989$, $p<0.001$, Fig.1).

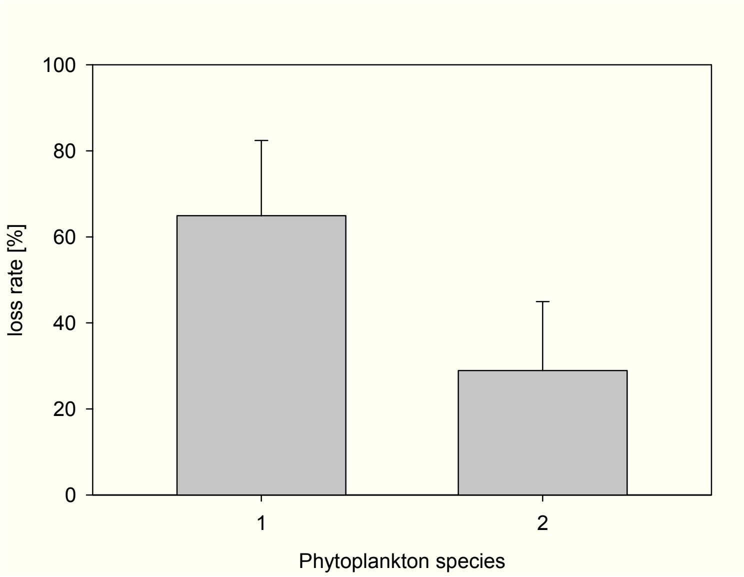

Fig. 1. Mean specific loss rate from membranes during the CARD FISH protocole. 1 = *Prorocentum micans*, 2 = *Rhodomonas sp.* Means are significally different. P < 0.001.

This result supported our assumptions that larger algae (Prorocentrum cell length = ca. 30 µm, Rhodomonas length = ca. 20 µm) get lost from the membranes at higher rates during the protocole. In a further similar test, we included the algae species *Prorocentrum micans*, *Emiliania huxleyi* (cell length = ca. 5 µm) and *Nitzschia sp*. (cell length = ca. 10 µm) and found significantly higher loss rates for the both larger species (data not shown).

In conclusion, we realized that due to the vulnerability of samples on membranes, only a very limited amount of samples could be analyzed in parallel. The observed relationship between cell size and loss rate during the incubation poses a severe problem to the analysis of mixed phytoplankton communities: Size-specific loss rates can deteriorate the real diversity of cell sizes originally present in the sample and can therefore lead to misinterpretations. The method should therefore only be used for plankton communities that constitute of species with similar ranges of cell size, or if foucus on a specific size class of phytoplankton is the study goal.

2.2 Hybridization of phytoplankton cells by the general eubacterial oligonucleotide probe EUB338

By following the protocole described by Medina-Sánchez et al. 2005, and utilising the general eubacterial oligonucleotide probe “EUB338”, we successfully stained brightly the majority of bacterial cells in samples taken from phytoplankton stock cultures (Fig 2).

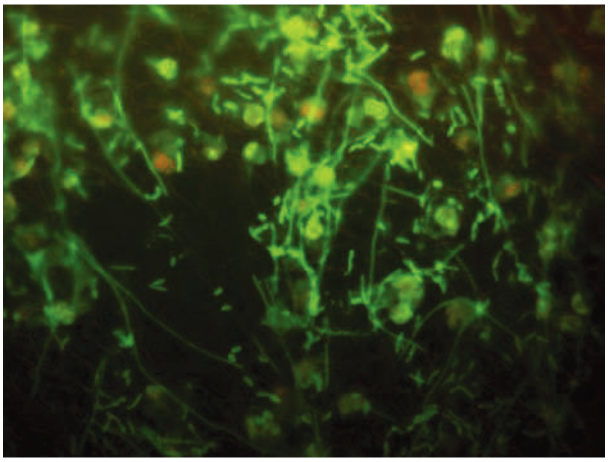

Fig. 2. Epifluorescence microphotopgraph of bacteria hybridized with EUB338 oligonucleotide probe (yellow) in a phytoflagellate culture. Phytoplankton cells in the background (red fluorescence of chlorophyll).

However, epifluorescence microscopic observations repeatedly showed that staining was not restricted to bacterial cells but occurred among phytoplankton cells from different laboratory species and from natural samples . Potential sources for failure of the laboratory method were excluded by strictly following the incubation protocole and strictly controlling incubation conditions. We assumed the EUB338 to match the 16S rRNA of chloroplasts of several common phytoplankton species due to the eubacterial phylogenetic origin of these organelles that developed by endosymbiosis of prokaryotes in eukaryotic cells. Using the probeCheck web page (department of Microbial Ecology, Univ. of Vienna) for the EUB338 oligonucleotide sequence gave theoretical support for our assumption. We found confirmation for our assumption by Biegala et al. 2005, who showed that 84 % of picoeukaryotes from natural coastal waters (in Brittany, France) were hybridised by EUB338.

Thus, the only two possible ways to apply (CARD) FISH for studying phagotrophy in phytoplankton are: Either to work exclusively on dinoflagellates which, accoding to our experiences, and available plastid sequence information (Biegala et al. 2002) should not to be affected by hybridization of EUB338, most probably due to their chloroplasts different phylogenetic origin by secondary endosymbiosis: or to choose oligonucleotide probes for specific bacterial subgroups, which do not match chloroplasts or mitochondria. However, the latter choice does not promis deep insight into the real picture of predator-prey relationships between algae and bacteria, since the abundance of species of bacterial subgroups is generally not representative for whole natural bacterial community abundances. Using subgroup specific probes would, if successfully applied, therefore most probably underestimate the importance of mixotrophy. Biegala et al. 2005 further applied the group specific eubacterial probes GAM42a and CF319a, that hybridize Gamma-proteobacteria and Cytophaga-Flavo-Bacteria, respectively, to her environmental plankton samples. Although the two probes were not expected to match picoeukaryotes due to their respective sequences, they hybridised 10 and 34 % of picoeukaryotes, respectively, yielding strong fluorescence of the eukaryotes. The authors supposed intracellular symbiotic or predatory bacteria inside the picoeukaryotes to be responsible for the unexpected labelling.

This points out a further limitation of FISH for the study of phagotrophy in general: Since this method can only be applied for staining dead and permeabilized cells, it cannot provide a causal explanation for the existence of labeled bacteria inside eukaryotic cells. Such bacteria may either be living endosymbionts of algae or dead, ingested prey.

3.) Resume: Towards an improved study of mixotrophy in phytoplankton

Due to the described methodological insufficiencies, the study of mixotrophy in phytoplankton, especially under field conditions, stays difficult and challenging. Nevertheless, modern molecular methods seem to be superior to the classical additions of labeled tracer prey particles, in that they do not interfere with the microbial ecosystem and in that in theory, they allow the study of potential specific feeding relationships between phytoplankton and bacterial phylogenetic groups. To reach these specific study aims and based on our experiences with CARD FISH for the study of mixotrophy, it would be crucial to further develop molecular probes that finally do not hybridize with algae any longer.

Concerning the high loss rates, particularly of large algae species, from membrane filters during the CARD FISH incubation procedures, an alternative methodology may be to carry out the hybridization in liquid plankton samples and to transfer the visualization of cells and detection of phagocytosed prey to a flow cytometer. However, even during preparation of CARD FISH for flow cytometric analysis, cell loss cannot be completely avoided (Biegala et al. 2003) and existing protocols would definitively have to be tested, and comprehensively adjusted to the study aims and the respective samples.

While flow cytometry provides a tool for rapid counting of (by size and fluorescence patterns) easily-discriminable phytoplankton groups or of phytoplankton monocultures, it cannot replace microscope based species- or genus-specific phytoplankton determination. However, once a particular species has been identified and sequenced, it may become possible to target and hybridize this (mixotrophic) phytoplankton species by a specific fluorescent-labeled oligonucleotide probe (Biegala et al. 2003). Subsequently, application of dye-labeled bacterial specific probes to that same sample may be possible in order to detect phagocytosed bacterial prey within cells of this species.

So, for a comprehensive study of mixotrophy in phytoplankton, reaching from environmental factors that promote feeding behaviour in different species to potential prey selectivity and quantification of grazing rates in the field, the combination of several traditional and modern methods and a cooperation of scientists from different biological disciplines would clearly be the ideal way.

CHAPTER 2

COMPOSITION AND CONCENTRATION OF DISSOLVED ORGANIC CARBON INTERACTIVELY AFFECT MIXOTROPHIC *OCHROMONAS MINIMA* POPULATION YIELD BY ALTERING SUPPLY WITH BACTERIAL PREY

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1.) Introduction

Phytoplankton and heterotrophic bacteria are major competitors for dissolved inorganic nutrients in aquatic habitats. Heterotrophic bacterial growth in freshwater and marine systems can be limited by dissolved inorganic nutrients and/or dissolved organic carbon (Elser et al. 1995, Jansson et al. 2006, Keiblinger et al. 2010). Dissolved organic carbon (DOC) constitutes the largest fraction of organic carbon in the ocean, and heterotrophic bacteria are its main consumers (Kirchman 2008). Increasing concentrations of biologically degradable DOC therefore provoke higher growth and nutrient consumption in carbon-limited, yet nutrient-replete bacteria. As a consequence in this situation, autotrophic phytoplankton growth declines with increasing availability of labile DOC, due to increasing shares of inorganic nutrients being bound by the bacterial fraction, as has been demonstrated in mesocosm experiments in polar and temperate coastal waters (Thingstad et al. 2008, Joint et al. 2002).

Global change alters the biogeochemical cycling of carbon in aquatic microbial communities. Recent studies indicate, in particular, a strong impact of global change phenomena on the coupling between autotrophic phytoplankton production and heterotrophic bacterial processes. Experimental studies with pelagic food webs have shown that warming increases heterotrophic bacterial production in phytoplankton blooms and have indicated shifts of organic carbon reservoirs from the particulate to the dissolved fraction (Hoppe et al. 2008, Kim et al. 2011, Wohlers-Zöllner et al. 2012). Concerning the effect of increasing atmospheric CO_2 concentrations on plankton communities, Riebesell et al. (2007) found natural phytoplankton communities consuming more dissolved inorganic carbon (DIC) in nutrient induced blooms at enhanced CO_2 concentrations. Since the increased carbon consumption, in their study, was not reflected in increased particulate carbon, the additional carbon is supposed to have entered the DOC pool by algal exudation. In addition to enhanced CO_2-levels and ecosystem internal changes in carbon fluxes, global change is supposed to globally affect northern coastal ecosystems in particular by increasing riverine loads of terrigenous dissolved material (tDOM) that originate from melting permafrost soils (Freeman et al. 2001, Wickland et al. 2012).

As has been shown by Thingstad et al. (2008), additional organic carbon can stimulate heterotrophic bacterial growth and thereby escape the planktonic system as CO_2 by bacterial respiration, at the same time negatively affecting phytoplankton biomass production and thus restricting the biological carbon pump. However, mixotrophy in planktonic protists is increasingly recognized as an important and wide-spread evolutionary strategy (Burkholder et al. 2008, Liu et al. 2009, Sanders and Gast 2011, Stukel et al. 2010, Zubkov and Tarran 2008). The capability to combine phototrophic and heterotrophic modes of nutrition provides organisms with several ecological advantages for energy and resource acquisition. Uptake of particles (phagotrophy) or dissolved organic compounds (osmotrophy) can substitute photosynthetic energy production or provide cells with essential growth factors, micro- and macronutrients if these are limiting in the inorganic dissolved pool (Glibert et al. 2012 and references therein, Maranger et al. 1998, Porter 1988). Ingestion of bacteria is a crucial way for mixotrophic phytoflagellates to acquire nutrients (Arenovski et al. 1995, Nygaard and Tobiesen 1993, Rothhaupt 1997, Stibor and Sommer 2003, Stukel et al. 2010), since bacteria usually compete better than phytoplankton for low dissolved nutrient concentrations (Kirchman 2008, Thingstad et al 1996).

However, mixotrophy itself is a multifaceted strategy, with some species being primarily phototrophs that are in addition capable of heterotrophic nutrition and with other species being placed rather on the heterotophic end of a behavioral continuum from strict autotrophy to strict heterotrophy (Jones 1997). Thus, photo- and heterotrophic processes in mixotrophs are regulated in species-specific ways by cell internal mechanisms and by a range of environmental factors, such as light intensity, inorganic nutrient concentrations or prey abundance (Granéli et al. 1999). In that way, mixotrophic phytoflagellates, that are bacterial competitors and, at the same time, function as bacterial predators, may differ substantially from purely autotrophic phytoplankton in their response to increasing concentrations of DOC. We hypothesized, firstly, that in plankton systems in which heterotrophic bacteria are limited by organic carbon, additional DOC increases mixotrophic phytoflagellate growth and abundance by increasing availability of the bacterial prey. Secondly, a mixotrophic strategy by which major nutrients (N and P) are taken up in form of bacterial prey could have the following implications for mineral supply ratios in the phytoflagellates. Biomass elemental N:P ratios of heterotrophic bacteria are variable and depend on growth substrate C:N:P stoichiometry, as well as on growth phase and bacterial community composition (Heldal et al. 1996, Makino et al. 2003, Makino and Cotner 2004, Vrede et al. 2000). N:P ratios of bacterial prey can thereby differ substantially from the Redfield Ratio of N:P for unlimited phytoplankton growth (Vrede et al. 2000). We therefore hypothesize that increasing DOC concentrations do not only increase mixotrophic phytoflagellate abundance, but in addition, influence the N versus P nutrient limitation pattern of the mixotrophs by altering nutrient supply ratios through the bacterial prey.

To test both our hypotheses, we conducted a laboratory experiment, using the mixotrophic phytoflagellate *Ochromonas minima* as a model species. We investigated the influence of DOC additions and the connected effect of bacterial prey abundance, on *O. minima* growth pattern and carrying capacity, on the efficiency of its photosystem II and on the nutrient limitation pattern of *O. minima* populations when having reached capacity. To create different bacterial prey abundances, non-axenic *O. minima* cultures were enriched with different concentrations and three different types of labile DOC, which differed in variety and composition of degradable organic compounds.

2.) Methods

2.1 Stock cultures and experimental setup

Ochromonas minima (Throndsen 1969) was pre-cultured for several weeks in semi-continious stock cultures in K-Medium (Keller and Guillard 1985, Keller et al. 1987) based on artificial seawater. Twenty percent of the culture volume was replaced by fresh medium every second day. Artificial sea water for the stock culture and experimental media was prepared using ultra-pure Milli-Q water in order to strictly exclude DOC from the fresh growth medium. To create sufficiently high start biomasses for the experiment, stock cultures were concentrated by centrifugation and re-suspended in nutrient reduced K-Medium (nitrogen 20 µmol l^{-1}, phosphoros 1,25 µmol l^{-1}). As was checked microscopically, the cells did not take any damage from the concentration procedure. The experiment was established in 400 ml polysterene cell culture flasks (Greiner Bio-One), growing phytoplankton under a 12:12 h day:night cycle under controlled light intensity and temperature conditions (65 µmol quanta PAR and 20° C). The experiment was started by a single initial addition of three different DOC-sources to the medium. Each DOC source was added in a gradient to result in final concentrations of 1.3, 2.6, 9.0 and 16.0 mg C l^{-1} and treatments were replicated 4 times. Twelve control treatments did not receive any DOC addition.

2.2 DOC enrichment

Stock solutions of the three different complex types of DOC were prepared by dissolving saccharose and a commercially available milk powder in artificial seawater. The simplest type of DOC was a pure solution of saccharose and the DOC type of intermediate complexity was a pure solution of milk powder. The most complex DOC type was prepared by a mix of saccharose and milk powder in a way that each of the two substrates delivered 50 % of total organic carbon. Thus, the main difference between DOC types was the portion of DOC originating from sugars (lactose and maltodextrins, from milk, and saccharose) relative to DOC from proteins and lipids (from milk).

Thus, the three DOC kinds also differed in the quantity of additional nutrients, since milk powder contains dissolved organically bound N (DON) and phosphoros. Table 1 sums up the concentrations of DON and P that are due to DOC additions of the two complex DOC types.

Table 1. DON and P additions by complex DOC enrichments

DOC [mg/l]	Intermediate complex DOC type		Most complex DOC type	
	DON [µmol/l]	P [µmol/l]	DON [µmol/l]	P [µmol/l]
1.3	3.3	0.26	1.65	0.13
2.6	6.6	0.52	3.3	0.26
9.0	22.8	1.82	11.4	0.91
16.0	40.6	3,24	20.3	1.6

2.3 Algal and bacterial abundance

Initial sampling (experimental day 0) was done in four replicates taken straight from the start culture before its distribution among treatment flasks. Subsequent sampling throughout the experiment was done from the experimental flasks. *O. minima* abundance was measured as chlorophyll a by in-vivo fluorescence (Trilogy Laboratory Fluorometer, Turner Designs). Quantum Yield measurements were performed with a pocket sized PAM fluorometer (Aqua Pen-C 100, Photon Systems Intruments) on *O. minima* cultures that had been stored for at least 10 minutes in darkness. Samples for bacterial abundance were fixed with glutaraldehyde (1,3 % final concentration), were shock frozen in liquid nitrogen and stored at -80˚ C until analysis. Bacterial counts were subsequently done on 200 µl of dilutions of thawed samples, stained with 2 µl SYBR green (stock dilution 1:100) for at least 15 minutes, using a high throughput bench top flow cytometer (guava easyCyte^{TM} HT, guava Technologies).

2. 4 Nutrient limitation bioassays

At day 30, each of the 60 experimental treatments was subsampled to assess the prevailing nutrient limitation of *O. minima*. Treatment replicate subsamples were distributed onto 4 15-ml-glassvials, and spiked with phosphoros (20 µmol l^{-1}), nitrogen (80 µmol l^{-1}), phosphoros and nitrogen in combination (20 µmol l^{-1}P, 80 µmol l^{-1} N), and sterile artificial seawater as a control, respectively. Bioassays were incubated at the experimental temperature and light conditions for 3 days and phytoplankton abundance was monitored daily by in vivo-fluorescence as described above. We used the statistical model selection procedure described by Andersen et al. (2007) to classify the type of nutrient limitation and followed Ptacnik et al. (2010) to summarize the categories N, P, or combined N and P limitation, in the one-dimensional indicator *L*, which scales between -1 and +1. L = + 1 means 100 % probability of P-Limitation, L = -1 means 100 % probability of N-Limitation, whereas L = 0 signalizes no *or* co-limitation (Ptacnik et al. 2010).

2.5 Statistics

Two-way-ANOVAs were calculated on *Ochromonas minima* final chlorophyll a concentrations and on maximum bacterial abundances to test on statistically significant differences between treatments provoked by the factors “DOC concentration” and “DOC source” and by the interaction of both factors. One-way-ANOVAs were calculated on *Ochromonas minima* final chlorophyll a concentrations within treatments groups that received the same DOC source to test on statistically significant differences between treatments caused by DOC concentration. All ANOVAs were performed using SigmaPlot 11.0.

3.) Results

3.1 *Ochromonas minima* growth dynamics

Ochromonas minima growth patterns over time were influenced by both the concentration and the type of dissolved organic carbon enrichment. In control treatments without any DOC addition, the flagellate population never entered into a logistic growth phase but instead, after an initial lag phase, slowly increased linearly until the end of the experiment (Fig. 1A).

***O. minima* growth with simple DOC source**

O. minima rapidly started to grow and performed typically sigmoid shaped growth curves at the two moderate DOC concentrations, i.e. 1.3 and 2.6 mg C l^{-1} (Fig. 1B and Fig. 1C). In contrast, in treatments that received 9.0 mg C l^{-1}, *O. minima* behaved very similarly to treatments that received no DOC (Fig. 1D). Flagellate growth was negative over the first 10 experimental days in treatments that received simple saccharose at the highest concentration (16.0 mg C l^{-1}, Fig. 1E), and subsequently started to slowly increase linearly.

O. minima growth with complex DOC sources

O. minima performed logistic growth in all treatments that received complex organic carbon additions (Fig. 1B-E).

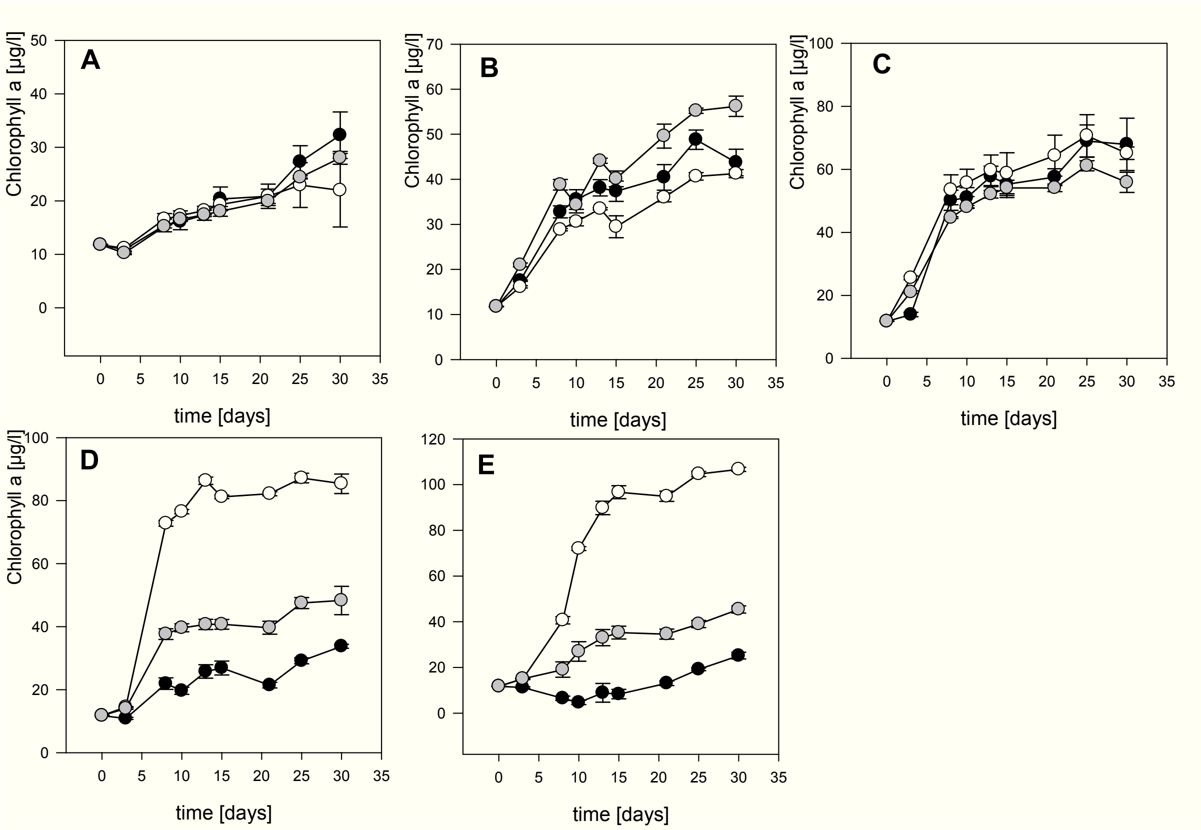

Figure 1.*Ochromonas minima* population growth timeseries

A no DOC addition, **B** 1.3 mg Cl^{-}, **C** 2.6 mg Cl^{-1}, **D** 9.0 mg Cl^{-1}, **E** 16.0 mg Cl^{-1}.

Black circles: simple DOC type, white circles: intermediate complex DOC type, grey circles: most complex DOC type. Error bars represent standard errors.

3.2 *Ochromonas minima* photosynthetic yield

Initial response of *O. minima* quantum yield

On experimental day 3, there was a general unimodal relationship between quantum yield and DOC concentration in all treatments. Highest efficiencies of photosystem II, on day 3, were found at the lowest carbon addition (1.3 mg C l^{-1}) among the treatments that received pure saccharose (Quantum yield mean = 0.62 ± 0.00 SE). In treatments that were spiked with both complex DOC sources, quantum yields peaked (mean = 0.63 ± 0.00 SE) at 2.6 mg C l^{-1} initial DOC additions (Fig 3).

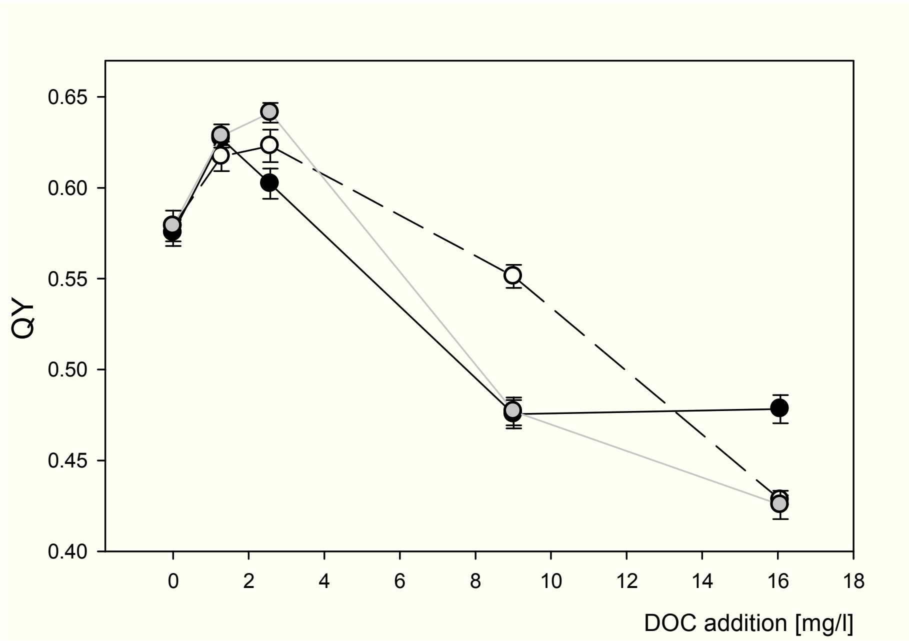

Figure 3.*Ochromonas minima* Quantum Yield (QY) on experimental day 3

Black circles: simple DOC type, white circles: intermediate complex DOC type, grey circles: most complex DOC type. Error bars represent standard errors.

Quantum Yield in control treatments

In control treatments, efficiency of *O. minima* photosystem II, measured as quantum yield, increased over the first three experimental days. During the rest of the experiment, quantum yield of control samples fluctuated around the initial start value (Fig. 2A).

Quantum Yield in treatments of the simple DOC source

Quantum Yields increased during the first three experimental days in treatments that received 1.3 and 2.6 mg C l^{-1} that originated from the simple DOC source (Fig. 2B and 2C). In treatments with 9.0 mg C l^{-1} initial DOC concentration, quantum yields fluctuated around the start value during the entire experimental period (Fig. 2D). Quantum yield decreased over the first 5 experimental days in treatments that received the highest (16 mg C l^{-1}) simple organic carbon addition and subsequently, photosynthetic performance recovered gradually during the rest of the experimental time (Fig. 2E).

Quantum Yield in treatments of the intermediate complex DOC source

In treatments that received 1.3 and 2.6 mgC l^{-1}, quantum yields increased over the first three incubation days and later varied around the experimental start value (Fig 2B and 2C). Photosynthetic efficiency increased only moderately during the first 3 experimental days in treatments that received 9 mg C l^{-1} but remained elevated up to day 7, after which photosynthetic performance of the flagellates decreased towards the end of the experiment (Fig. 2D). In treatments receiving 16.0 mg C l^{-1}, quantum yields decreased initially up to day 3, recovered until day 10, reaching moderately high values, and finally decreased during the remaining experimental period (Fig. 2E).

Quantum Yield in treatments of the most complex DOC source

Quantum yield increased over the first three experimental days in treatments that initially received 1.3 and 2.6 mg C l^{-1} (Fig. 2B and 2C). Photosynthetic efficiency did not show any clear trend in treatments that received 9 mg C l^{-1} but fluctuated around start conditions during the entire experiment (Fig. 2D). Experimental units that initially received 16 mg C l^{-1} of the most complex carbon source behaved almost identically to those treatments that received an equal concentration of the intermediate DOC source (Fig. 2E).

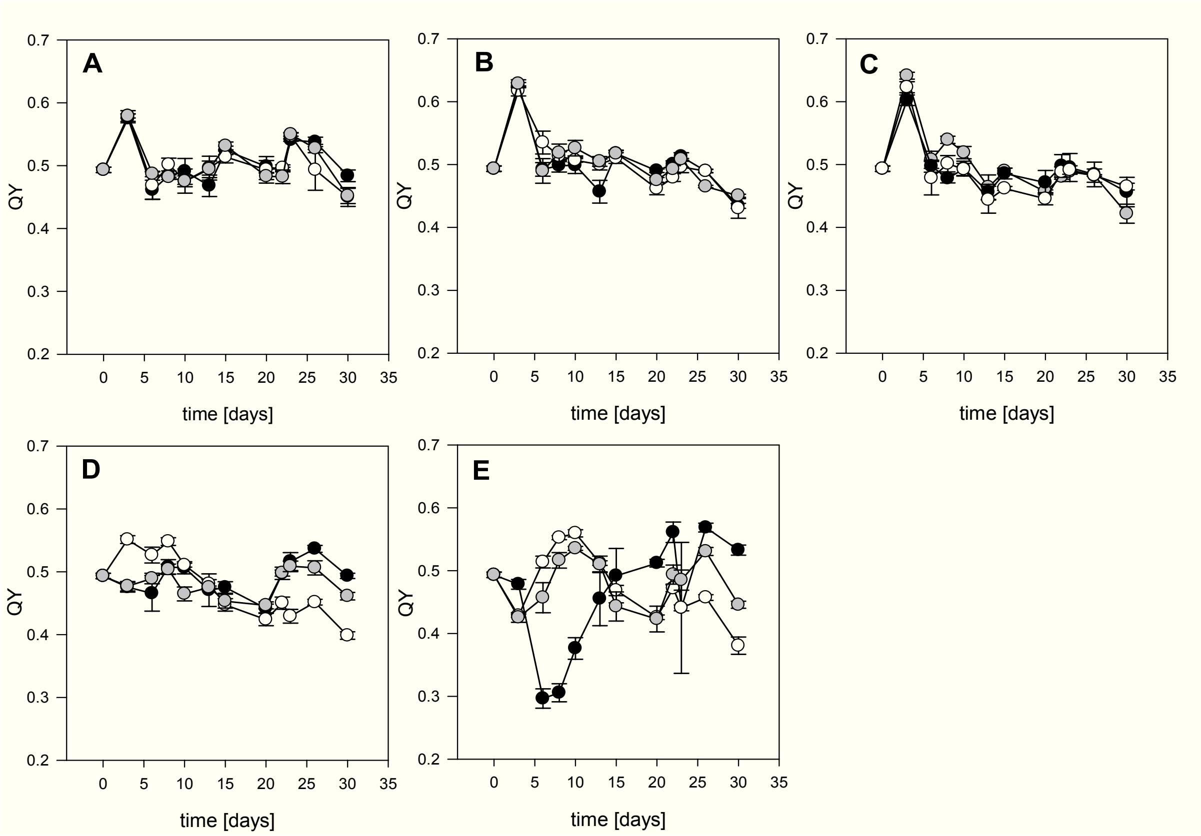

Figure 2.*Ochromonas minima* Quantum Yield (QY) timeseries

A no DOC addition, **B** 1.3 mg Cl^{-}, **C** 2.6 mg Cl^{-1}, **D** 9.0 mg Cl^{-1}, **E** 16.0 mg Cl^{-1}.

Black circles: simple DOC type, white circles: intermediate complex DOC type, grey circles: most complex DOC type. Error bars represent standard errors.

3.3 *Ochromonas minima* maximum abundance

Maximum algal abundance at carrying capacity (experimental day 30) was significantly influenced by both initial DOC concentration ($F_{4,45} = 44.91$, $p < 0.001$) and DOC type ($F_{2,45} = 55.46$, $p < 0.001$) and the interaction between both factors ($F_{8,45} = 35.43$, $p < 0.001$).

O. minima maximum abundance with simple carbon source

The concentration of the simple carbon source significantly influenced *O. minima* maximum abundance ($F_{4,15} = 13.970$, $p < 0.001$, table 3) *O. minima* capacity biomasses followed a hump shaped distribution over the initial DOC concentration gradient, with highest biomasses (mean chlorophyll-a concentration 67.88 µg l^{-1} ± 8.31 SE) reached for 2.6 mg C l^{-1} added carbon (Fig. 4).

Maximum abundance with intermediate complex carbon source

There was a significant effect of initial DOC concentration of the intermediate complex carbon source on *O. minima* carrying capacity ($F_{4,15} = 91.270$, $p < 0.001$, table 3). There was a positive, non-linear relationship between initial DOC concentration and maximum algal biomass. In treatments that received 16 mg C l^{-1}, *O. minima* reached the highest final abundance of the entire experiment (mean chlorophyll a-concentration = 106.68 µg/l ± 0.94 SE) (Fig. 4).

Maximum abundance with most complex carbon source

Phytoflagellate yield was significantly influenced by the concentration of the most complex carbon source ($F_{4,15} = 16.863$, $p < 0.001$, table 3). *O. minima* abundance was higher in treatments that received organic carbon, however, there was no further linear positive relationship between DOC concentration and maximum phytoflagellate yield (Fig. 4).

Table 3. One-factorial ANOVA results for maximum abundances of *Ochromonas minima.*

Dependent variable	**Source of variation: DOC concentration**	
O. minima abundance with *simple DOC source*	$F_{4,15}$ =13.970	$p < 0.001$
O. minima abundance with *more complex DOC source*	$F_{4,15}$ = 91.270	$p < 0.001$
O. minima abundance with *most complex DOC source*	$F_{4,15}$ = 16.863	$p < 0.001$

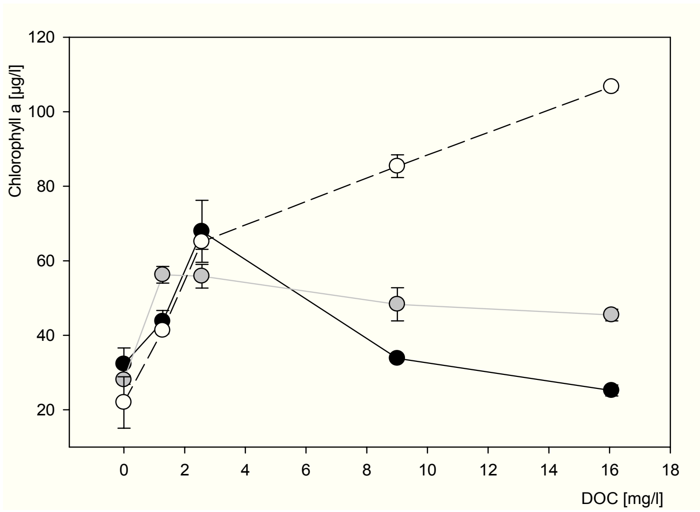

Figure 4.*Ochromonas minima* carrying capacities on initial DOC additions.

Black circles: simple DOC type, white circles: intermediate complex DOC type, grey circles: most complex DOC type. Error bars represent standard errors.

3.4 Bacterial abundance patterns

Maximum bacterial abundances were significantly positive related to initial DOC concentration ($F_{4,45}$ = 1365.55, p < 0.001) and influenced significantly by DOC source ($F_{2,45}$ = 927.30, p < 0.001). There was a significant interaction of DOC concentration and DOC source ($F_{8,45}$ = 285.00, p < 0.001, table 2)

Highest measured bacterial concentrations among treatments were reached in those experimental units that had received the highest concentration of intermediate complex organic carbon (mean cells/l = $29.47*10^6$ ± 447161 SE). These treatments still contained the highest bacterial abundances among all experimental treatments on day 30 (mean cells/l = $5.50*10^6$ ± 238396 SE) (Fig 5E). On day 30, bacterial concentrations were still higher in most of the DOC treatments than in treatments that had received no DOC addition.

Bacterial abundance on the simple organic carbon source

Bacterial abundances rapidly increased at the start of the experiment and reached a first peak at day 1 (concentrations 1.3 and 2.6 mg C l^{-1}, fig. 5B and 5C) or day 3 (concentrations 9 and 16 mg C l^{-1}, fig. 5D and 5E). Peaks were thereafter followed by a crash of bacterial populations and a subsequent slow increase of bacterial numbers towards the end of the experiment (concentrations 1.3-9.0 mg C l^{-1}, fig. 5B-5D). Treatments that received 16 mg C l^{-1} DOC showed constantly elevated bacterial abundances from day 3 until the end of the experiment (fig 5E).

Bacterial abundance on the intermediate complex organic carbon source

Bacterial abundances reached a first peak of abundance on day 1 (concentrations 1.3-9.0 mg C l^{-1}, fig. 5B-5D) or day 3 (16 mg C l^{-1}, fig. 5 E). Populations subsequently crashed, and the growth dynamic continued by slow increase until the experimental end (concentrations 1.3 – 9 mg C l^{-1}, fig. 5B-5D) or by relatively small fluctuations of bacterial numbers (16 mg C l^{-1}, fig. 5 E).

Bacterial abundance on the most complex organic carbon source

Bacterial abundances reached a first peak of abundance on day 1 (concentrations 1.3 and 2.6 mg C l^{-1}, fig. 5B and 5 C) or day 3 (concentrations 9 and 16 mg C l^{-1}, fig. 5D and 5E). After population crashes, abundances slowly increased towards the experimental end (concentrations 1.3 – 9 mg C l^{-1}, Fig. 5B-5D) or fluctuated little (16 mg C l^{-1}, fig. 5E).

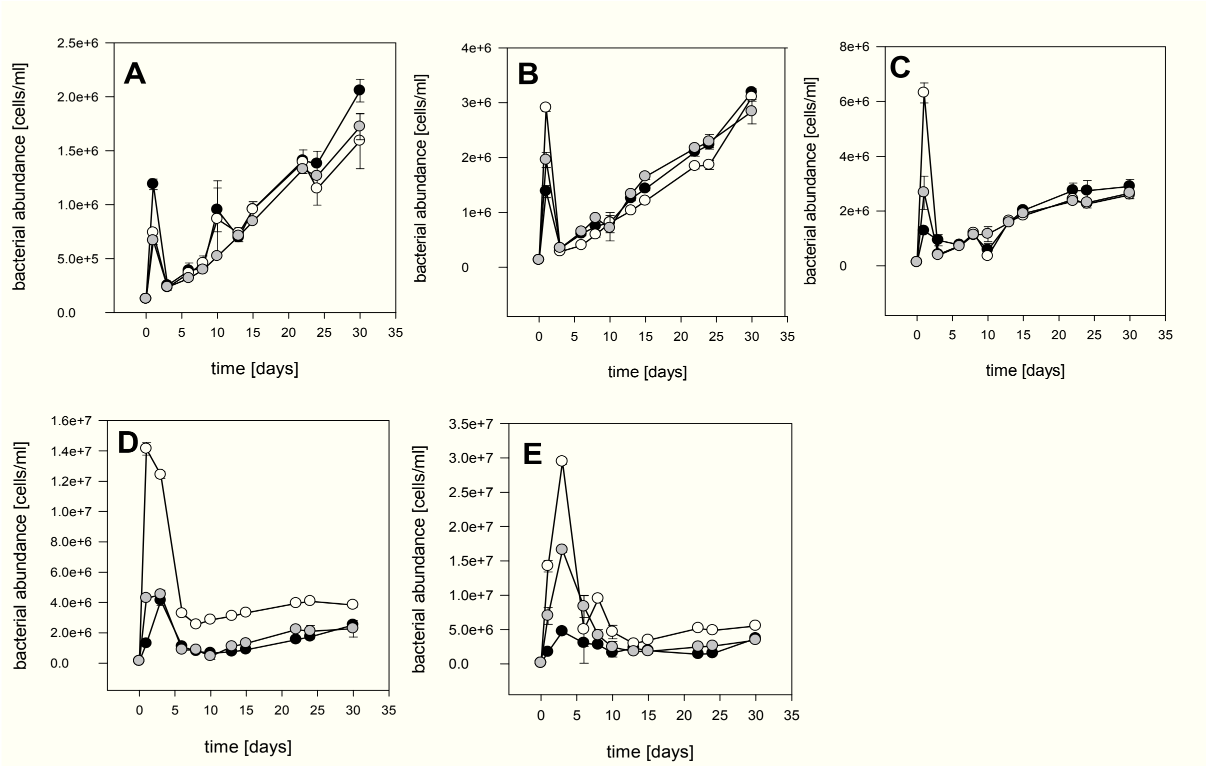

Figure 5.Heterotrophic bacteria abundance time series.

A no DOC addition, **B** 1.3 mg Cl^{-}, **C** 2.6 mg Cl^{-1}, **D** 9.0 mg Cl^{-1}, **E** 16.0 mg Cl^{-1}.

Black circles: simple DOC type, white circles: intermediate complex DOC type, grey circles: most complex DOC type. Error bars represent standard errors.

Table 2. Two-factorial ANOVA results for maximum abundances of*Ochromonas minima* and bacteria.

Dependent variable	**Source of variation**		
Maximum abundance	DOC concentration	DOC type	interaction
O. minima	$F_{4,45} = 44.91$ P < 0.001	$F_{2,45} = 55.46$ P < 0.001	$F_{8,45} = 35.43$ P < 0.001
bacteria	$F_{4,45} = 1365.55$ P < 0.001	$F_{2,45} = 927.30$ P < 0.001	$F_{8,45} = 285.00$ P < 0.001

3.5 *Ochromonas minima* nutrient limitation pattern

While final phytoflagellate biomass was strongly influenced by initial DOC additions, our results show no differences in the final nutrient limitation pattern of the flagellates among treatments. The univariate limitation indicator L, in our experiment, takes clear negative values and scales mainly to -1, without any clear relationship to the concentration of the organic carbon addition, nor to the type of DOC added, and thus indicates pure nitrogen limitation for most treatments (Fig. 6).

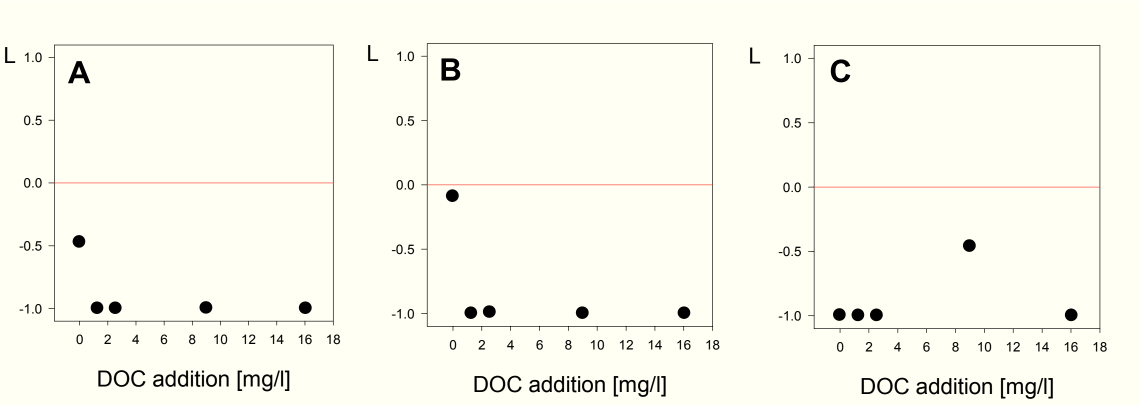

Figure 6.Univariat Limitation Indicator “L “, derived from bioassays with *Ochromonas minima*.

Asimple DOC source**, B** intermediate complex DOC source**, C** most complex DOC source.

L = + 1: 100 % probability of P-Limitation, **L = -1** : 100 % probability of N-Limitation,**L = 0** : no *or* co-limitation (Ptacnik et al. 2010)

4. Discussion

DOC Supply and Bacteria-Algae Coupling

Our first hypothesis was that increasing concentrations of DOC should increase mixotrophic phytoflagellate abundance by increasing availability of bacterial prey.

Our first hypothesis was partly supported by our experimental results. All DOC additions up to 2.6 mg C l^{-1} boosted *Ochromonas minima* growth about 2-fold, whereas, the effect of DOC on phytoflagellate growth at higher added concentrations depended on the kind of organic substrate. While our simple sugar-DOC source provoked renewed reduction of phytoflagellate growth when given at concentrations higher than 2.6 mg C l^{-1}, the most complex organic carbon substrate provoked a saturation of phytoflagellate yield. It was only by adding the intermediate complex DOC source, that we managed to sustain increasingly higher flagellate yields with increasing DOC supply.

Interestingly, the short-term effect of DOC on photosynthetic performance of *O. minima* differed substantially from the long term effect on flagellate biomass yield. Carbon additions of 1.3 and 2.6 mg C l^{-1}, initially resulted in a higher efficiency of the photosystem II, but higher DOC concentrations initially suppressed photosynthetic efficency, compared to controle treatments. It is therefore surprising that we found sustained higher *O. minima* abundances at high complex DOC concentrations.

This discrepancy can be explained as follows. We suppose the positive effect of lower DOC concentrations on photosynthetic performance is provoked by better resource provisioning of the phytoflagellate cells, due to increased uptake of more abundant bacteria. Better resource provisioning of phytoplankton is reflected in higher quantum yields (Falkowski and Raven 2007), which we also found when giving small and intermediate DOC concentrations. Our results are partially in line with those found by Flöder et al. (2006). For *Ochromonas minima*, the authors have shown that net population growth is light-dependent, even though the flagellate can survive at extremely low light intensities and is ingesting bacteria under illuminated as well as dark conditions. As the population growth rate of the flagellate in their study depended on the number of bacterial prey available, and the onset of flagellate population growth depended on a threshold of bacterial concentration, irrespective of the trophic state of the experimental system, the authors suggested that *O. minima* gains nutrients from ingested bacteria (Flöder et al. 2006).

Consequently, regarding resource utilization, there is strong support that *O. minima* can be seen functionally as a light-dependent predator, which depends to a high extend on particulate nutrients and its population growth rate and capacity should be controlled by availability of bacterial prey and light intensity. In previous experiments we confirmed, that *O. minima* growth and carrying capacity is positively related to light intensity (de Schryver et al., unpublished results). Our view of bacteria as an essential nutrient source for *O. minima* is most impressively supported by our results for treatments that received up to 2.6 mg C l^{-1} saccharose. Increasing bacterial abundances with increasing DOC should have lowered the dissolved inorganic nutrient pool available to flagellates, but nonetheless, flagellate populations increased with increasing DOC levels. This is consistent with observations from a study on a different mixotrophic *Ochromonas* species, which differs from *O. minima*, in that its growth is not light-dependent. In line with our results, the addition of glucose to bacteria-containing, nutrient-deplete cultures of this *Ochromonas* species substantially enhanced the flagellates growth (Sanders et al. 2001). In addition, recent reports by Schmidtke et al. (2006) on a naturally occurring *Ochromonas sp*., that dominates the plankton biomass of an extremely acidic lake, underline its ecological importance as an efficient bacterial grazer. The authors estimated that *Ochromonas sp*. in their study lake is grazing daily a mean of 88 % (epilimnion) and 68 % (hypolimnion) of the single-celled bacterial production. Our results are, however, in contrast to those found by Thingstad et al. (2008) for natural arctic phytoplankton communities in a DOC-addition mesocosm experiment. As their communities consisted on overall of diatom species, which are competitors, but no predators of heterotrophic bacteria, we suggest, that DOC and increased bacterial growth resulted in decreased phytoplankton abundance in their experiment. Consequently, this means, that the relative abundance of mixotrophic species in a phytoplankton community should have, in general, a high influence on the community´s response to changes in environmental factors (DOC, nutrients) that basically affect the microbial food web.

Nevertheless, in our microcosm experiment, photosynthesis of the phytoflagellate was initially negatively affected for higher concentrations of DOC and higher bacterial abundances. We explain this by the osmotic stress that high concentrations of monosaccharids cause for phytoplankton. With starting growth of bacteria and their consumption of sugars, osmotic conditions for algae should have improved with time, however. After this initial stress phase, *O. minima* in treatments with higher DOC concentrations should have profited from the enhanced bacterial prey abundance and reached higher standing stocks than without DOC provision.

However, *O. minima* end biomasses decreased in treatments that received high saccharose concentrations. While the phytoflagellates were initially inhibited by negative osmotic conditions shortly after the DOC addition, rapidly growing bacteria depleted the medium for nutrients, while consuming the nutrient-free DOC source. Thus, bacterial biomass in saccharose-treatments should have become limited by nutrients, quickly, and the early peak in bacterial abundance, provoked by the single DOC addition, should have rapidly broken down. Consequently, *O. minima,* which is assumed to be grazing inefficiently while osmotically stressed, is supposed to have been limited by prey abundance on the long run, in treatments that received saccharose-DOC. This is reflected in the falling flagellate biomass yields for very high added concentrations.

Contrary, offering high concentrations of complex DOC that also contains organically bound N, provides bacteria a better growth substrate. Treatments that received DON and dissolved P from the DOC source reached higher bacterial peak abundances, which can be explained by a release of bacteria from nutrient limitation and therefore more complete consumption of DOC (Elser et al. 1995). Also, the more complex DOC sources should have supported a more diverse bacterial flora and higher bacterial densities due to the higher diversity of exploitable growth substrates. High phytoflagellate biomasses in treatments with high concentrations of complex DOC can therefore be explained by a bottom-up effect in the bacteria-flagellate food chain, as has been demonstrated previously for strictly heterotrophic flagellates (Pengerud et al. 1987). This effect was larger when the intermediate complex carbon source was added, since additional P, enhanced and prolonged bacterial population growth.

Utilization of DOM directly by phytoplankton

An increasing number of phototrophic protists from diverse habitats are found able to utilize dissolved organically bound nutrients (Anderson et al. 2002, Fagerberg et al. 2010, Glibert and Legrand 2008). Current research demonstrated the use of dissolved organic nitrogen that enters aquatic systems by effluents and from soil wash out (Carlsson et al. 1999, Davidson et al. 2012, Fagerberg et al. 2009, Kamjunke and Tittel 2008, Liu et al. 2011). Our experimental design cannot completely eliminate this possible nutritional pathway for *O. minima*. However, we did not find any differences between the effects that our three types of DOC in concentrations up to 2.6 mg C l^{-1}provoked in *O. minima* maximum biomass yield, nor in its growth pattern. If *O. minima* used additional DON, which was provided by amino acids in our more complex substrates, it should have used the ambient amount of DOC even more efficiently, which should have resulted in higher biomass yields on DOC substrates other than saccharose. We therefore do not suppose that DON is an important nutrient source for *O. minima.*

Effects of DOC on nutrient limitation of phytoplankton

In our second hypothesis we claimed that DOC concentration should have an influence on the N vs. P nutrient limitation pattern of *O. minima*, due to changes in particulate nutrient supply by bacterial prey.

Our experiment did not give any results supporting our second hypothesis, since *O. minima* in all treatments was limited by N. Yet, our results fit with observations from phytoplankton in Scandinavian, DOC-rich humic lakes. In these lakes, phytoplankton is often dominated by mixotrophic taxa, a fact, which is supposed to be caused by high DOC concentrations and high abundance of bacterial prey favoring phagotrophic species. Experimental studies in these lakes revealed that inhabitant mixotrophs are often limited by N, and over-provisioning of the mixotrophic phytoflagellates due to feeding on P-rich bacterial prey is suggested as an explanation for these observations (Jansson et al. 1996, Jones 2000). However, N-limitation may not be the only and ultimate possibility concerning effects of nutrient supply ratios in mixotrophs. More recent studies demonstrate a broad variability in bacterial C:N:P ratios, depending on growth conditions and bacterial species composition, as well as on species specific homeostatic regulation of C:N:P content (Heldal et al. 1996, Makino et al. 2003, Makino and Cotner 2004, Vrede et al. 2000).

Therefore, more research that focuses elemental ratios both in bacteria and in mixotrophs is needed to elucidate the probably more complex relationship between N:P:DOC-supply, bacterial stoichiometry and nutrient supply to mixotrophic phytoflagellates. In addition, bacterial growth activity in general can impact the stoichiometry of the dissolved nutrient pool and thus is able to influence in another way the nutrient limitation of phytoplankton (Danger et al. 2007), thereby further complicating the picture.

Importance of DOC and plankton mixotrophy for biogeochemical cycling

DOC is still an underestimated factor in phytoplankton ecology. DOC may be of increasing importance to mediate food web processes and to affect the composition of the microbial food web in pelagic ecosystems under global change pressure.

In particular, the production and fate of DOC has a crucial role on the CO_2 balance between atmosphere and ocean. Increasing CO2 concentrations in future ecosystems may lead to higher inorganic carbon fixation by phytoplankton. However, the additional carbon will only be transported and long-term stored in biomass of deeper water layers of the oceans, if it is not degraded and respired already in the upper mixed ocean layers. Recent experimental results indicate a possibly higher DOC production by algal exudation in a high CO_2 world, and a subsequent increased formation of transparent exopolymer particles (TEP) that enhance aggregation and therefore particle sinking and may thereby increase the efficiency of the biological carbon pump (Riebesell et al. 2007)

Our results demonstrate that another possible fate of the additionally produced DOC in the future ocean may be the transport through the microbial loop, which means an ultimate transport to higher trophic levels (Azam et al. 1983), and thus more CO_2 re-entering the atmosphere. On the other hand, our results also indicate that phytoplankton community composition may shift to mixotrohic species which could in theory re-bind respired CO_2 from bacteria in photosynthesis, while grazing on bacteria to gain mineral nutrients. Interestingly, Egge et al. (2009) found a tendency of increased cumulative primary production at elevated CO_2 partial pressure in the 5-10 µm phytoplankton fraction in a mesocosm experiment with natural communities. Initial blooms of diatoms in their experiment were followed by blooms of *Emilliania huxleyi* and other nano- and pico-phytoplankton species, among which, at least in theory, may have been mixotrophic species to a large extend, given the high importance of this functional group of phytoplankton (Burkholder et al. 2008, Liu et al. 2009, Zubkov and Tarran 2008).

Similar effects on phytoplankton community composition have been found in studies investigating the role of ocean warming. Sommer and Lengfellner (2008) found a tendency towards smaller phytoplankton mean size and phytoflagellate dominated spring blooms in warmer experimental mesocosms. Along with higher bacterial densities in warmer mesocosms, these findings reasonably allow to speculate about a high portion of these flagellates being mixotrophs.

More future experimental investigations should therefore focus on the interactive role of global change scenarios and phytoplankton functional diversity on carbon biogeochemistry and carbon balance of the atmosphere and oceans. We conclude from our results that further studies should also take into account the carbon and nutrient stoichiometry in the dissolved fraction, because of its crucial role on bacterial and phytoplankton dynamics, as revealed by our study. We conclude that photosynthetic microorganisms of the microbial loop may be key players in changing aquatic ecosystems but with the broad diversity of the mixotrophic strategy, more research is needed to address the role of single mixotrophic species´ characters for biogeochemical cycling.

5.) Acknowledgements

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CHAPTER 3

INFLUENCE OF DISSOLVED ORGANIC CARBON ON GROWTH AND COMMUNITY COMPOSITION OF PHYTOPLANKTON ASSOCIATED WITH BACTERIA

Vera de Schryver and Kristina Röben

I. Introduction

There is growing concern about the consequences of global change for ecosystem services of coastal waters. Under future climate scenarios, precipitation is going to increase in the northern hemisphere. As a consequence, increased run-off is going to drain catchments and transport higher land-derived nutrient loads into coastal areas, leading to eutrophication of marine ecosystems. Simultaneously, and especially in the northern regions (e.g. the northern Baltic Sea Area), terrigenous dissolved or suspended organic material is increasingly washed out of soils and transported to the seas. In Arctic regions, this development is going to be even more enhanced due to global warming and the connected melting of permafrost soils (Freeman et al. 2001, Wickland et al. 2012). In general, warming of surface waters in marine ecosystems is strongly affecting the biogeochemical cycling of carbon through microbial food webs. In particular, warming was found to affect heterotrophic bacterial processes stronger than autotrophic processes in phytoplankton (Hoppe et al. 2008). In addition, experimental studies on warming in marine planktonic systems indicated shifts of organic carbon pools from the particulate to the dissolved fraction (Kim et al. 2011, Wohlers-Zöllner et al.) Concerning plankton ecosystem structure, a general shift towards smaller sized species was found to be caused by warming (Daufresne et al. 2009, Peter and Sommer 2012). In addition, warming has been shown to provoke diverse and complex changes in the temporal abundance patterns of phytoplankton (Sommer and Lengfellner 2008, Suikkanen et al. 2013 Taucher et al. 2008).

Phytoplankton and heterotrophic bacteria are the basal producers in planktonic food webs that provide energy and elements for all higher trophic levels, either by flow through the classical food chain from autotrophic phytoplankton directly to metazoan zooplankton, or through the microbial food web. At the same time, phytoplankton and planktonic heterotrophic bacteria are main competitors for dissolved inorganic nutrients. Heterotrophic bacteria are the main consumers of dissolved organic carbon (DOC) in the oceans (Kirchman 2008) and consequently, growth of heterotrophic bacteria, in freshwater as well as in marine systems can be limited by dissolved inorganic nutrients and/or dissolved organic carbon (Elser et al. 1995, Jansson et al. 2006, Keiblinger et al. 2010). Increasing concentrations of biologically available DOC therefore provoke higher growth and nutrient consumption in carbon-limited, yet nutrient-replete bacteria. However, in this situation, autotrophic phytoplankton growth declines with increasing availability of labile DOC, since increasing shares of inorganic nutrients get consumed by the bacterial fraction (Thingstad et al. 2008, Joint et al. 2002).

Thus, both warming and increasing concentrations of terrigenous organic carbon (TDOC) in eutrophic planktonic systems favor heterotrophic bacterioplankton production and negatively affect autotrophic phytoplankton production due to shortness of available nutrients. Nevertheless, there is growing awareness of the fact, that a major part of planktonic protists in freshwater and marine ecosystems adopts a mixotrophic nutritional strategy, rather than being pure auto- or heterotrophs (Burkholder et al. 2008, Liu et al. 2009, Flynn et al. 2013). Combining phototrophic and heterotrophic modes of nutrition in one species, mixotrophic protists gain ecological advantages for resource and energy acquisition if these are limiting in the environment. Phagotrophy of prey particles (e.g. bacteria or diverse eukaryotic cells) can substitute energy production if light availability is insufficient for photosynthesis or can deliver micro- and macronutrients in oligotrophic environments (Glibert et al. 2012 and references therein, Maranger et al. 1998, Stibor and Sommer 2003). It was recently shown in a mixotrophic phytoflagellate species, that warming can stimulate the heterotrophic process components in mixotrophs (e.g. grazing on bacterial prey) more strongly than the photosynthetic functions, turning them into stronger heterotrophs under warmer conditions (Wilken et al. 2012).

Being competitors and predators of bacteria at the same time, mixotrophic phytoflagellates may differ substantially from purely autotrophic phytoplankton in their response to increasing concentrations of tDOC and warming of sea surface waters. We come to the following hypotheses:

1.) Increasing concentrations of DOC in eutrophic systems should favor mixotrophic phytoflagellate growth and abundance by increasing availability of heterotrophic bacterial prey, whereas strictly autotrophic phytoplankton species should decline due to increased bacterial competition for dissolved inorganic nutrients.
2.) Warmer temperature should enhance the relative importance of mixotrophs in a phytoplankton community, irrespective of DOC concentration.

We tested our hypotheses in a laboratory experiment, using the mixotrophic phytoflagellate *Ochromonas minima* as a model species. We investigated its growth along gradients of dissolved organic carbon, both in bacteria containing monocultures, and as part of an artificial laboratory community, comprised of a total of 6 species and their associated bacteria. We studied the effect of warming by replicating DOC treatments at two different incubation temperatures.

II. Methods

II.1 Phytoplankton stock cultures and experimental setup

The mixotrophic model species *Ochromonas minima* and 5 common phytoplankton species were pre-cultured individually for several weeks in semi-continuous stock cultures in f/2-medium based on artificial seawater of low salinity (18 psu). Artificial sea water for the stock culture and experimental media was prepared using ultra-pure Milli-Q water, in order to strictly exclude DOC from the fresh growth medium. In order to achieve high start biomasses for the experiment, stock cultures were concentrated by centrifugation and re-suspended individually in nutrient reduced f/2-medium (nitrogen 20 µmol l^{-1}, phosphorus 1,25 µmol l^{-1}), prior to the start of the experiment.

Phytoplankton communities were created by adding aliquots of all six individual monocultures to freshly prepared, nutrient reduced (nitrogen 20 µmol l^{-1}, phosphorus 1,25 µmol l^{-1}) growth medium. Based on microscopic biovolume determination of the individual species (Hillebrand et al. 1999), communities were established in a way that each species contributed an equal biovolume. *O. minima* monocultures were established following the same procedure. The experiment was run in 400 ml polysterene cell culture flasks (Greiner Bio-One) and phytoplankton was grown under a 16:8 h light:dark cycle under controlled light intensity (100 µmol quanta PAR). Distributed among four incubators, communities and *O. minima* were cultured at two different mean temperatures (14 and 18° C +/- 1° C during light/dark cycles) and in a concentration gradient of initially added labile DOC (saccharose - final concentrations 0.0, 0.5, 1.0, 2.3 and 3.6 mg C l^{-1}). Phytoplankton community treatments were replicated four times, whereas *O. minima* monoculture treatments were run in duplicates. Phytoplankton cultures were mixed twice a day, by gently inversing the culture bottles. All culture bottles were newly distributed among the incubators, daily, and, at this occasion, the position of the bottles inside the incubator was determined randomly.

II.2 Algal and bacterial abundance

Initial sampling (experimental day 0) was done in four replicates taken straight from the start community and the *O. minima* monoculture, respectively, before their distribution among treatment flasks. Subsequent sampling throughout the experiment was done from the experimental flasks.

Chlorophyll a concentration was measured fluorometrically after chlorophyll a extraction of glass fiber filter samples (Whatman GF/F) in 90 % acetone (Turner fluorometer) at the start and end of the experiment.

Samples for bacterial abundance were fixed with formaldehyde, were shock frozen in liquid nitrogen and stored at -80˚ C until analysis. Bacterial counts were subsequently done on dilutions of thawed samples, stained with SYBR green for at least 15 minutes, using a flow cytometer (FACSCalibur; Becton Dickinson).

II.3 Phytoplankton community composition and diversity

Phytoplankton community composition was determined at the end of the experiment from samples fixed with Lugol´s iodine by using the Utermöhl quantification method (Utermöhl 1958). Biovolume of individual species was calculated according to Hillebrand et al.

II.4 Statistics

Two-way-ANOVA was calculated on final heterotrophic bacteria abundances from phytoplankton communitiy-treatments to test on statistically significant differences between treatments provoked by the factors “DOC concentration” and “temperature” and by the interaction of both factors (SigmaPlot 11.0).

Linear regressions were calculated on data from 14° C- and 18° C-treatments, respectively, to test on linear relationships between DOC concentration and ln-transformed data of total community chlorophyll a-concentration, of bacterial end concentration in *O. minima* cultures, and of relative biovolume contributions of the different algae species to the community (Sigma Plot 11.0). To further test on an influence of temperature on these parameters, and/or an interactive influence by temperature and DOC, we used the "homogeneity of slopes design" of the general linear model module of the Statistica software (Statistica 8.0).

III. Results

III.1 Bacterial abundances

III.1.1 Bacterial abundances in *Ochromonas minima* cultures

Bacterial abundances increased from the start of the experiment in all experimental treatments and reached peak values on days two or four. Peak bacterial numbers tended to be higher in 18° C than in 14° C treatments. At the end of the experiment, cell concentrations did not differ from start concentrations, except in treatments that received 0.5 mg C l^{-1}, where cell concentrations were higher on day 7 than on day 1, and higher in 18° C than in 14° C treatments. For all other treatments, there was no effect of temperature on bacterial numbers at the experimental end (figure 1).

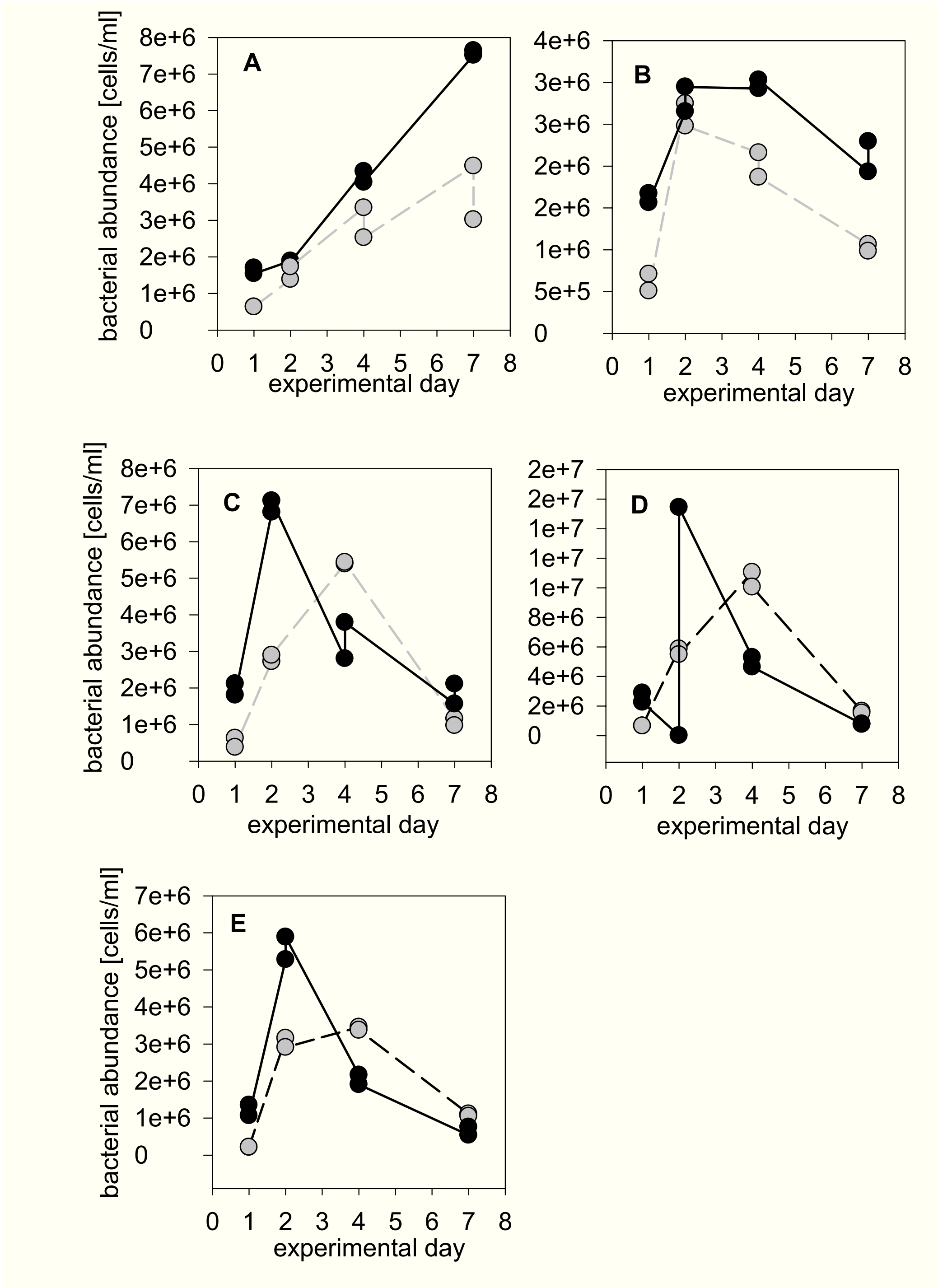

Figure 1. Heterotrophic bacteria abundance timeseries from *Ochromonas minima* cultures

A no DOC addition, **B** 0.5 mg Cl^{-}, **C** 1.0 mg Cl^{-1}, **D** 2.3 mg Cl^{-1}, **E** 3.6 mg Cl^{-1}.

Black circles: 18°C. Grey circles: 14°C. Error bars represent standard deviations.

Bacterial abundances at the end of the experiment in 18°C-treatments were about four-fold higher when no DOC was added, compared to treatments that received DOC. In these treatments there was a tendency towards decreasing bacterial cell numbers with increasing DOC additions. In 14°C- treatments, bacterial numbers were about two-times higher in treatments without DOC, whereas there was no further effect of increasingly higher DOC concentrations (figure 2).

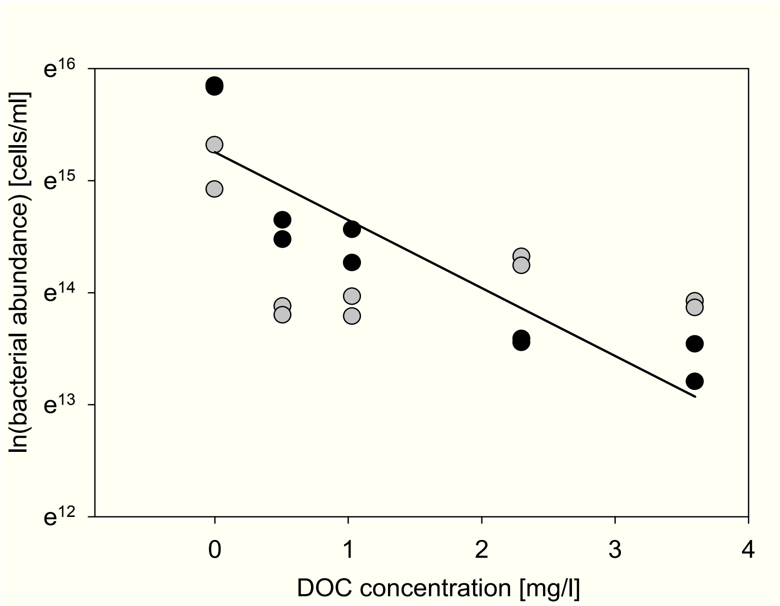

Figure 2 Heterotrophic bacteria abundances at the end of the experiment, dependent on DOC addition in *Ochromonas minima* cultures.

Black circles: 18°C. Grey circles: 14°C. Error bars represent standard deviations.

Regression line shown for significant linear regression in the 18 °C-data subset.

III.1.2 Bacterial abundances in phytoplankton communities

Bacterial abundances increased from the start of the experiment in all experimental treatments and reached peak values on days two or four in most of the treatments. Treatments that received 0.5 mg C l^{-1} at 18° C reached maximum bacterial numbers already on day 1, and treatments that received 1.0 C l^{-1} at 14° C showed peak values on day 7. However, no data are available on day 1 for these treatments, due to sample loss. There was no clear effect of temperature on peak bacterial abundances. At the end of the experiment, in 14° C treatments, there was a tendency for bacterial concentrations being higher than at the start of the experiment. For the warmer treatments, in general, there was no difference between start and end bacterial abundances, expect in treatments that received no DOC and 2.3 mg C l^{-1}, where bacterial numbers were lower at the end (figure 3).

At the end of the experiment, there was a significant interaction of DOC and temperature on bacterial concentrations. However, in treatments grown at 18° C, there was only a weak tendency for higher bacterial numbers in treatments that received DOC compared to treatments without any addition. For treatments grown at 14° C, the relationship between DOC addition and end bacterial concentrations was unimodal, with peak bacterial numbers for additons of 1.0 and 2.3 mg C l^{-1} (figure 4).

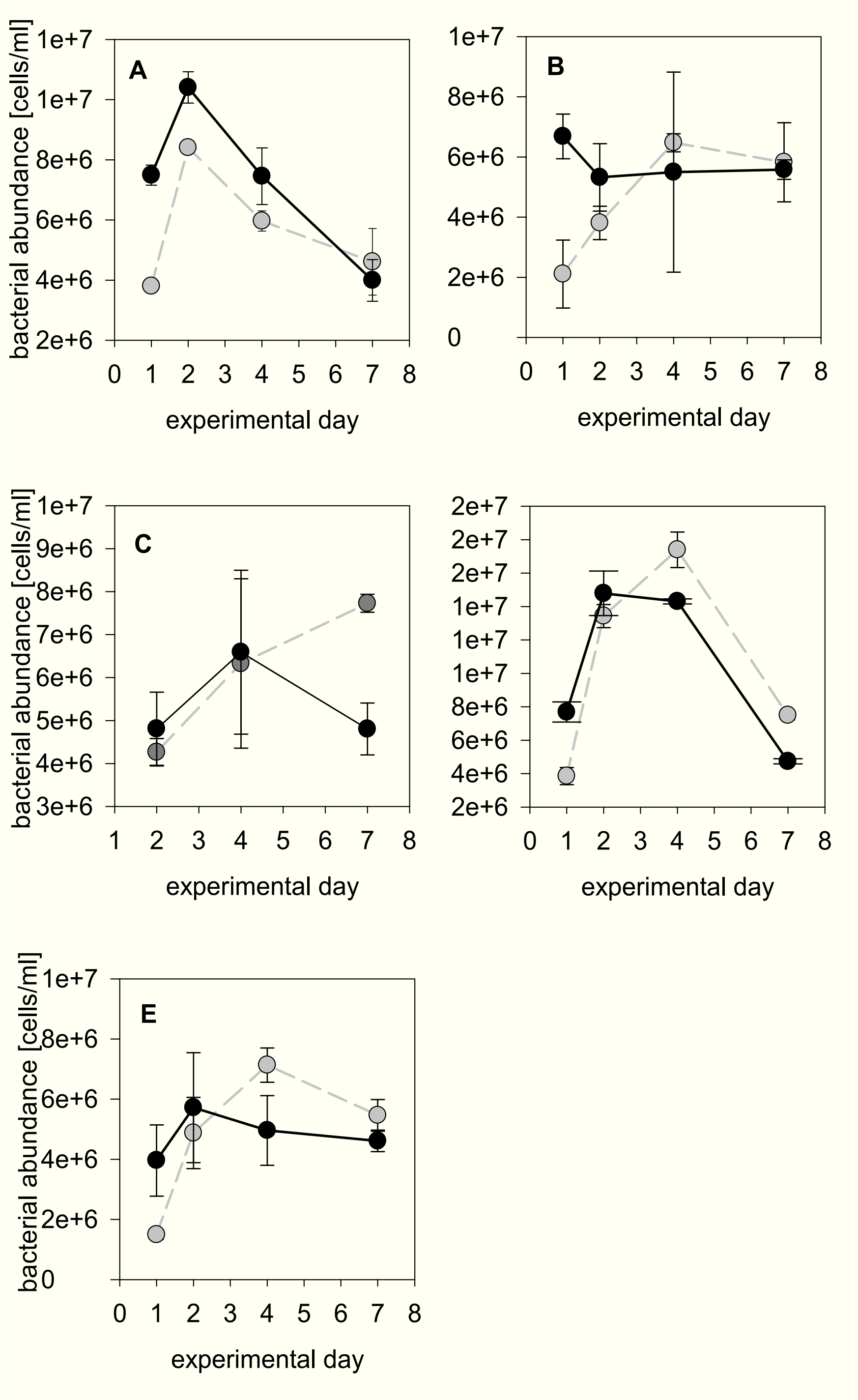

Figure 3. Heterotrophic bacteria abundance timeseries from Phytoplankton community cultures

A no DOC addition, **B** 0.5 mg Cl^{-}, **C** 1.0 mg Cl^{-1}, **D** 2.3 mg Cl^{-1}, **E** 3.6 mg Cl^{-1}.

Black circles: 18°C. Grey circles: 14°C. Error bars represent standard deviations.

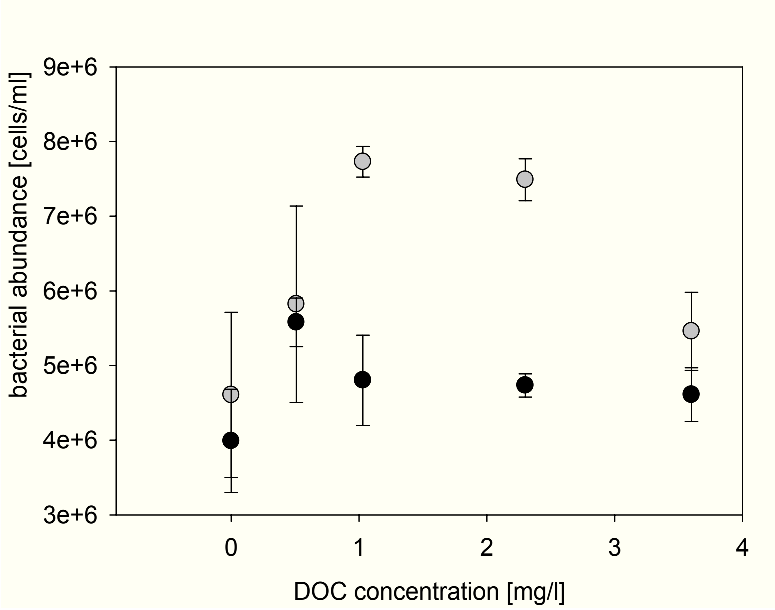

Figure 4. Heterotrophic bacteria abundances at the end of the experiment, dependent on DOC addition in Phytoplankton community cultures

Black circles: 18°C. Grey circles: 14°C. Error bars represent standard deviations.

III.2 Phytoplankton abundance and community structure

III2.1 *Ochromonas minima* monocultures

At the end of the experiment, under both incubation temperatures, *Ochromonas* abundance was maximal when DOC was added in concentrations of 0.5-2.3 mg C l^{-1}. In general, *O. minima* achieved higher densities when grown at 18° C compared to 14° C, and this difference in growth was higher for treatments that received 0.5-2.3 mg C l^{-1} (figure 5A).

A

III.2.2 Phytoplankton community cultures

Phytoplankton abundance – Chlorophyll a

At the end of the experiment and for both temperatures, phytoplankton biomass, measured as chlorophyll a concentration, declined drastically and exponentially with increasing DOC addition. Ln-transformation of the data thus yielded a significant linear decline of chlorophyll with increasing DOC under both incubation temperatures. (figure 5A). Chlorophyll a concentrations were significantly higher at 18° C than at 14° C though all DOC concentrations (Table 3).

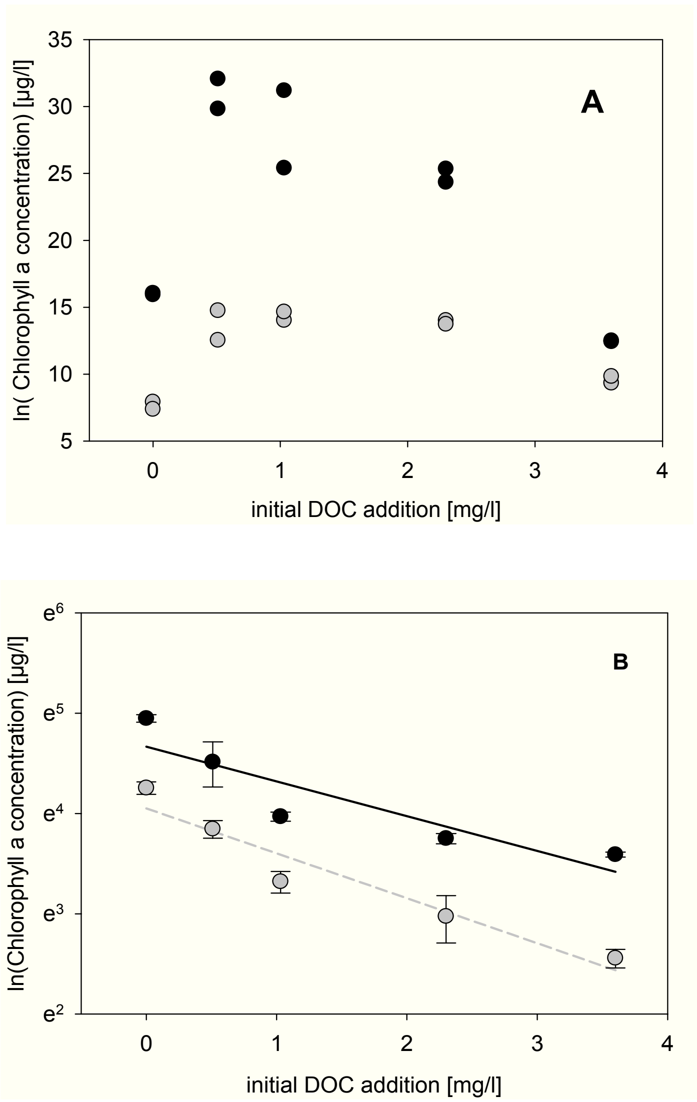

Figure 5. Phytoplankton abundance at the end of the experiment, dependent on DOC addition in **A** *Ochromonas minima* and in **B** phytoplankton community cultures.

Black circles: 18°C. Grey circles: 14°C. Error bars represent standard deviations.

Regression lines are shown for significant linear regressions.

Table 3. Statistics results from testing (by use of a general linear model) for significant influences of DOC (continuous variable), Temperature (categorical variable) and its interaction, on the response variables: final bacterial abundance (in *O. minima* cultures), chlorophyll-a concentration (communities), and relative biovolume contributions of the different algae species. Level of significance: $p < 0.05$

dependent variable	**Source of variation**		
	DOC concentration	**temperature**	**interaction**
ln(BA) (in *O. minima*)	**P < 0.001**	**P = 0.023**	**P = 0.015**
ln(Chl a) (communities)	**P < 0.001**	**P < 0.001**	P < 0.076
Ochromonas rBVC	**P < 0.001**	**P = 0.033**	P = 0.076
Thallassiosira rBVC	**P < 0.001**	P = 0.724	P = 0.670
Rhodomonas rBVC	**P < 0.001**	**P = 0.012**	P = 0.095
Prasinocladus rBVC	P = 0.559	P = 0.881	P = 0.463
Prorocentrum rBVC	**P < 0.001**	P = 0.280	P = 0.264
Alexandrium rBVC	**P < 0.001**	P = 0.061	P = 0.825

Phytoplankton community structure

Response of phytoplankton to DOC addition and temperature was species-specific. There was no measurable effect of both factors on the community biovolume of *Prorocentrum minimum*. *Alexandrium sp*. showed a tendency towards increasing biovolumes at higher DOC concentrations, which was not affected by temperature. There was a significant interaction of DOC and temperature on biovolumes of *O. minima* and *Thalassiosira sp*. (Table 1). In general, *Thalassiosira sp.*, and *Rhodomonas sp*. strongly lost in abundance with increasing DOC concentrations. In contrast, *O. minima* in phytoplankton communities showed no clear dependence on DOC concentration at 14° C, but at 18° C, its biovolume performed a unimodal distribution, similar to what we found for its monocultures. (Fig. 6).

Both dinoflagellate species contributed only minor to overall community biovolume through all DOC concentrations and for both temperatures. Nevertheless both dinoflagellate species´ relative biovolumes were significantly positively influenced by DOC, but not affected by temperature. *Thallassiosira sp*. and *Rhodomonas sp.* were the dominant species of the community when no DOC was added, whereas *Ochromonas minima* and *Prasinocladus sp*. contributed similar to community biovolume. Over the DOC gradient, the relative contribution of *Thallassiosira sp.* and *Rhodomonas sp.* diminished significantly in importance, whereas *O. minima* contributed increasingly to overall community biovolume and came to dominate the community in the highest DOC addition treatments. *O. minima´*s relative importance was singnificantly positively influenced by temperature and DOC. While the relative biovolume of *Thallassiosira* was not affected by temperature, the relative contribution of *Rhodomonas* to the phytoplankton community declined more drastically when it was incubated at the colder temperature. There was no effect of any of the experimental factors on *Prasinocladus* relative biovolume (figure 7, Tables 2 and 3).

Table 1. Two-factorial ANOVA results for heterotrohic bacterial abundance in phytoplankton communities at the end of the experiment and for absolute biovolumes of phytoplankton species. BV = Biovolume. Level of significance: $p < 0.05$.

	Source of variation		
dependent variable	**DOC concentration**	**temperature**	**interaction**
bacterial abundance	$F_{4,30} = 11.94$ **P < 0.001**	$F_{1,30} = 48.99$ **P < 0.001**	$F_{4,30} = 7.17$ **P < 0.001**
Ochromonas BV	$F_{4,30} = 6.84$ **P < 0.001**	$F_{1,30} = 140.66$ **P < 0.001**	$F_{4,30} = 11.17$ **P < 0.001**
Thallassiosira BV	$F_{4,30} = 61,26$ **P < 0.001**	$F_{1,30} = 80.17$ **P < 0.001**	$F_{4,30} = 19.28$ **P < 0.001**
Prasinocladus BV	$F_{4,30} = 4.10$ **P = 0.009**	$F_{1,30} = 8.99$ **P = 0.005**	$F_{4,30} = 1.66$ P = 0.184
Rhodomonas BV	$F_{4,30} = 25.95$ **P < 0.001**	$F_{1,30} = 11.28$ **P = 0.002**	$F_{4,30} = 2.58$ P = 0.057
Prorocentrum BV	$F_{4,30} = 0.36$ P = 0.82	$F_{1,30} = 2.08$ P = 0.15	$F_{4,30} = 1.96$ P = 0.12
Alexandrium BV	$F_{4,30} = 3.93$ **P = 0.011**	$F_{1,30} = 0.01$ P = 0.90	$F_{4,30} = 0.22$ P = 0.92

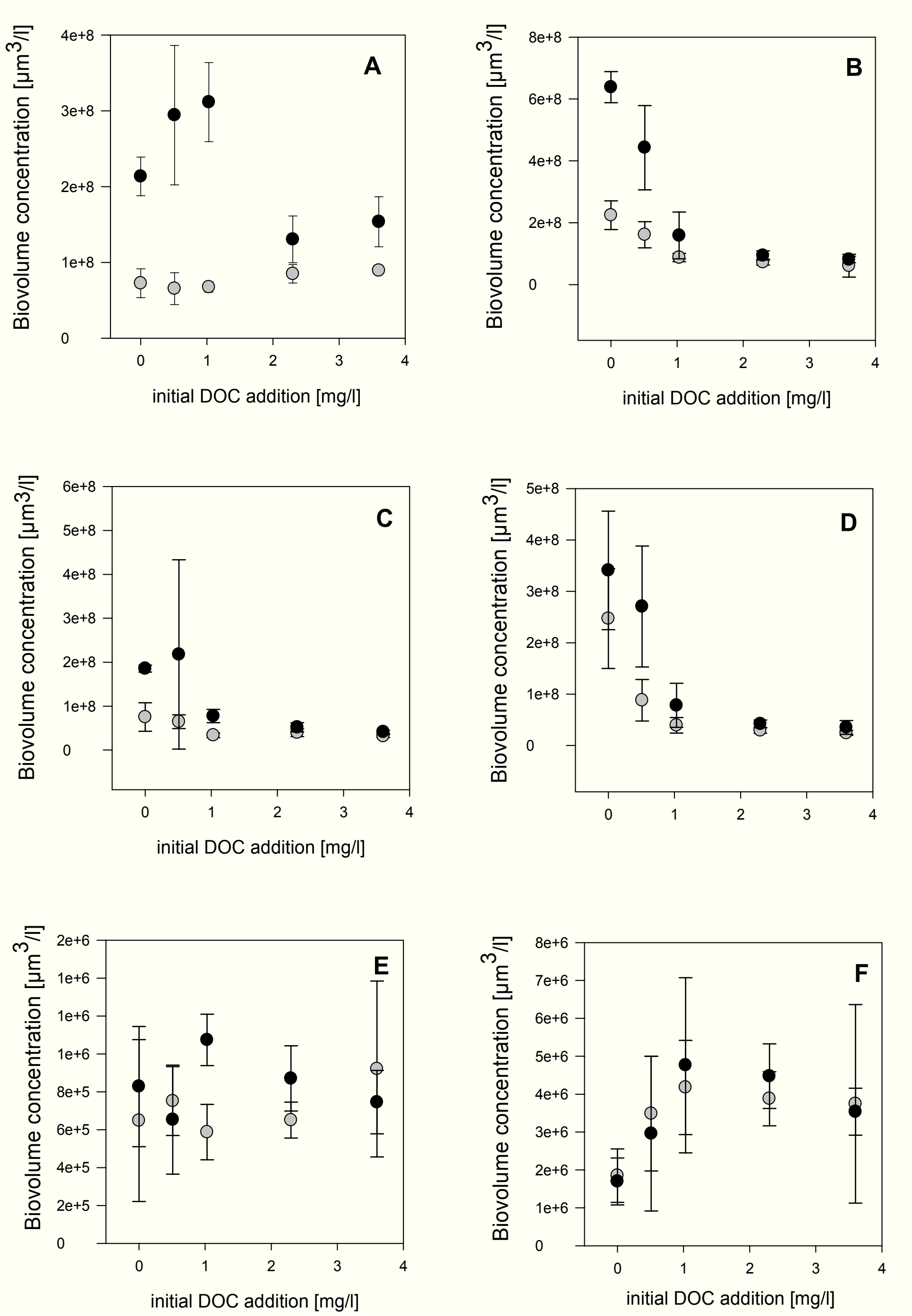

Figure 6.Biovolume concentrations of individual species in phytoplankton communities.

A *Ochromonas minima***,** **B** *Thalassiosira sp*., **C** *Prasinocladus marinus*, **D** *Rhodomonas sp*., **E** *Prorocentum minimum*, **F** *Alexandrium sp*.

Black circles: 18°C. Grey circles: 14°C. Error bars represent standard deviations.

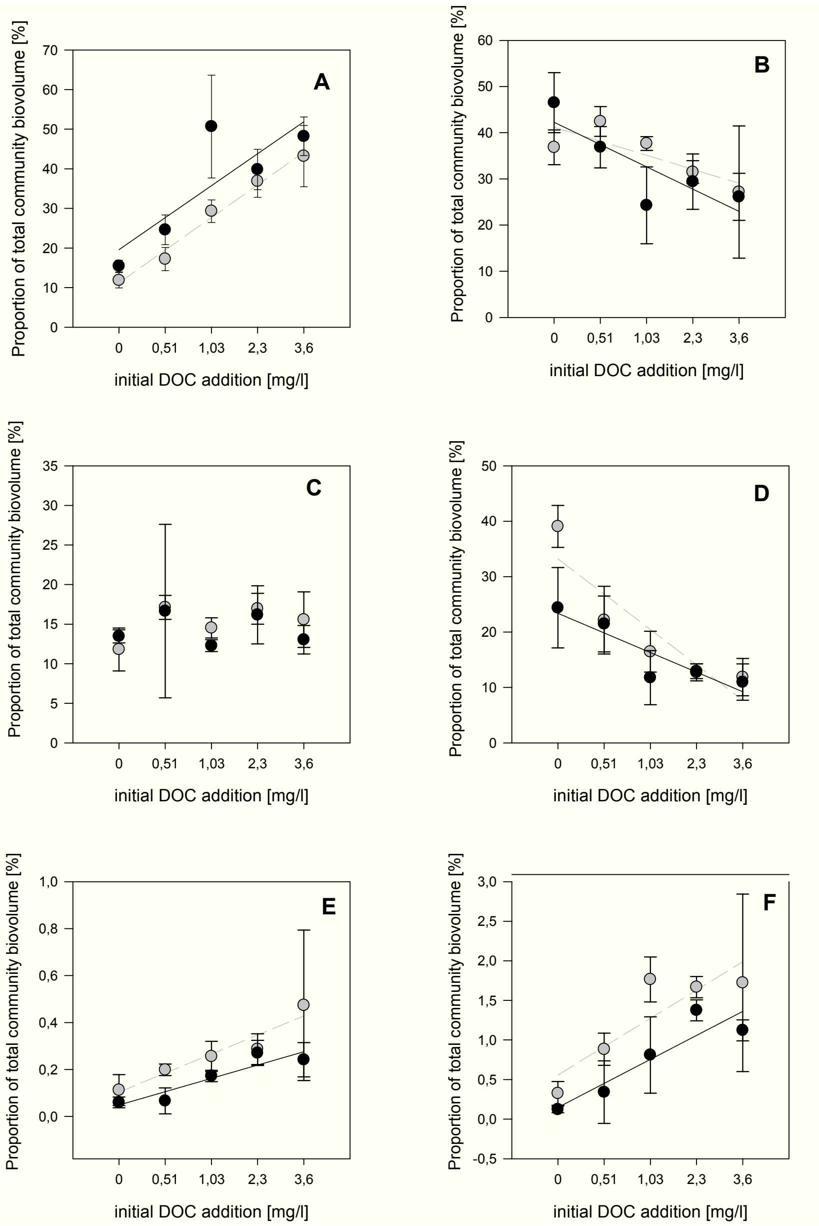

Figure 7. Relative Biovolume concentrations of individual species in phytoplankton communities.

A *Ochromonas minima*, **B** *Thalassiosira sp*., **C** *Prasinocladus marinus*, **D** *Rhodomonas sp*., **E** *Prorocentum minimum*, **F** *Alexandrium sp*.

Black circles: 18°C. Grey circles: 14°C. Error bars represent standard deviations.

Regression lines are shown for significant linear regressions.

Table 2. Linear regression statistics for relationships between DOC and the response variables final bacterial abundance (in *O. minima* cultures), chlorophyll-a concentration (communities), and relative biovolume contributions of the different algae species. Regressions statistics are calculated for 14°C and 18°C treatments, respectively. Temp. = Temperature. BA = bacterial abundance. Chl a = Chlorophyll a concentration. DOC = DOC concentration. rBVC = relative biovolume contribution. Level of significance: p < 0.05.

dependent variable	Temp.	equation	R^2	p
BA	18° C	ln(BA) = 15.25 – (0.60*DOC)	0.80	**< 0.001**
(in *O. minima*)	14° C	ln(BA) = 14.45 – (0.17*DOC)	0.20	0.195
Chl a	14° C	ln(chl a) = 4.05 – (0.45*DOC)	0.90	**< 0.001**
(communities)	18° C	ln(chl a) = 4.67 – (0.35*DOC)	0.79	**< 001**
Ochromonas	14° C	rBVC = 14.86 + (8.62*DOC)	0.83	**0.001**
rBVC	18° C	rBVC = 24.5 + (7.51*DOC)	0.43	**0.002**
Thallassiosira	14° C	rBVC = 40.47 - (3.60*DOC)	0.34	**0.006**
rBVC	18° C	rBVC = 39.17 - (4.39*DOC)	0.34	**0.007**
Rhodomonas	14° C	rBVC = 29.55 - (6.11*DOC)	0.56	**<0.001**
rBVC	18° C	rBVC = 21.38 - (3.41*DOC)	0.41	**0.002**
Prasinocladus	14° C	rBVC = 14.19 + (0.67*DOC)	0.09	0.183
rBVC	18° C	rBVC = 14.49 - (0.07*DOC)	0.00	0.93
Prorocentrum	14° C	rBVC = 0.13 + (0.08*DOC)	0.42	**0.002**
rBVC	18° C	rBVC = 0.07 + (0.05*DOC)	0.61	**< 0.001**
Alexandrium	14° C	rBVC = 0.77 + (0.33*DOC)	0.34	**0.006**
rBVC	18° C	rBVC = 0.29 + (0.30*DOC)	0.56	**< 0.001**

V. Discussion

V.1 Hypothesis 1

We hypothesized, firstly, that increasing concentrations of added DOC should promote growth of the mixotrophic model species *Ochromonas minima*, while purely autotrophic phytoplankton species should be negatively affected by DOC.

Considering *O. minima*, our results confirm the hypothesis, with the flagellate achieving higher standing stocks at elevated DOC concentrations and strongly reduced bacterial abundances in these treatments. However, the positive effect of DOC was reduced for increasingly high concentrations. This may be explained by increasing competition between the phytoflagellate and bacteria for some common essential dissolved growth factor (like e.g. a special vitamin) that *O. minima* may not be able to extract from bacterial prey. Our results are in line with those of Sanders et al. (2001) who studied a different mixotrophic *Ochromonas* species that also performed better, when glucose was added to the growth medium. Regarding the further five phytoplankton species which were included in our study, their reaction to increasing levels of added DOC was species specific. *Thalassiosira sp.* and *Rhodomonas sp.*, both very productive species, strongly reduced growth and confirmed our hypothesis. *Prasinocladus marinus* and *Prorocentrum minimum* seemed to have out-weighed the nutrient-depletion caused by increased bacterial abundances in DOC-rich treatments, whereas *Alexandrium* sp. showed a weak tendency towards better growth with higher DOC concentrations. Nevertheless, both our dinoflagellate species performed unexpectedly weak, and results should be considered with caution. Thus, the overall phytoplankton community response to enhanced DOC was mainly driven by the responses of *O. minima, Thalassiosira sp. and Rhodomonas sp.*. *O. minma* could not increase its productivity strong enough to offset the losses in phytoplankton biomass caused by the diatom and the cryptophyte. Thus, overall response of the phytoplankton community to increasing

DOC levels was a decline in biomass, which is in line with results from an Arctic mesocosm experiment, in which a small *Thalassiosira* species dominated communities (Thingstad et al. 2008). However, *O. minima* strongly gained in relative importance for the overall phytoplankton biovolume along the DOC gradient, finally contributing more than 40 % of total, in treatments with highest DOC concentrations.

Our findings fit well together with an experimental study on the potential of invasion of a mixotrophic *Ochromonas sp.* into established microbial communities that consisted of algae and heterotrophic protists (Katechakis and Stibor 2006). This is since, in our study, *Ochromonas minima* came to dominate communities when dissolved nutrient levels (although not measured) should have been lowest because of rapid consumption by carbon-replete, heterotrophic bacteria. Katechakis and Stibor (2006) demonstrated that *Ochromonas tuberculata* gained a competitive advantage from its mixotrophic strategy and successfully invaded and finally dominated planktonic microbial communities exclusively under oligotrophic conditions, that restricted specialist autotrophic phytoplankton´s success. By establishing a DOC gradient under constant dissolved nutrient concentrations in our study (i.e. by creating different DOC-to-nutrient ratios), we step-wisely deliberated heterotrophic bacteria from carbon limitation, which should have resulted in a trophic gradient, with low dissolved nutrient concentrations in treatments that received high DOC concentrations and which finally got dominated by *Ochromonas minima*.

V.2 Hypothesis 2

Our second hypothesis claimed that warmer temperatures should enhance the relative importance of mixotrophs in the phytoplankton community.

Most of our phytoplankton species, including *O. minima* experienced at least a trend towards higher biomass production in the warmer treatments at low DOC additions. In addition, *O. minima´*s contribution to total community biovolume was in fact positively influenced by temperature, but we have to judge this result with caution: Since the flagellate reached very high absolute abundances at warmer temperature and at intermediate DOC concentrations, also its relative contribution was raised the most, at a DOC concentration of 1.0 m C l^{-1}. The biovolume contributions of the other five single species were mainly unaffected by temperature (with exception of *Rhodomonas sp.*) and overall phytoplankton standing stock at the end of the experiment was significantly positive influenced both by warm temperature and falling DOC concentrations. We therefore, from our community treatment results, have only minor support for our hypothesis, that mixotrophs should increase in importance at higher temperatures. In general, phytoplankton response to warming is species specific (Huertas et al. 2011, Taucher et al. 2012). While declining primary productivity was found in warmer treatments in mesocosms with southern Baltic Sea phytoplakton (Hoppe et al. 2008), a long term analysis on three sub-basins of the northern Baltic Sea revealed an increase in total phytoplankton biomass explained by warming (Suikkanen et al. 2013). In the latter study, Cyanophyceae, Prymnesiophyceae and Chrysophyceae increased, wheas Cryptophyceae decreased with warming. Nevertheless, Wilken et al. (2012) demonstrated for a different *Ochromonas sp.* that, when grown in heterotrophic mode, its growth rate accelerated strongly with temperature, and its heterotrophic and mixotrophic growth rate was consistently higher than growth rate under autotrophic culture mode. However, mixotrophy is a broad behavioral strategy and the relative contributions of the heterotrophic and autotrophic modes to total nutrition vary strongly between species (Jones 1997). In *Ochromonas minima,* photosynthesis and phagotrophy both seem to be essential for growth, since the flagellate fails to grow in the dark (even with high bacterial concentrations present), it fails to grow without a certain threshold of bacterial prey, and it increases growth with increasing light intensity (Flöder et al. 2006, de Schryver et al. unpublished results). So far, no studies are available that quantify the respective importance of temperature on the flagellate´s heterotrophic and autotrophic parts of nutrition. However, results from our monocultures show, that the growth of the phytoflagellate is more positively affected by temperature under moderate DOC concentrations than under low DOC concentrations, indicating a potential positive effect of temperature on its heterotrophic nutrition mode.

V.3 Choice of experimental phytoplankton species

In our study we aimed to test for differences in growth between five autotrophic algae species and the mixotrophic model species *Ochromonas minima*. Mixotrophy, in general, means the combination of autotrophic and heterotrophic modes of nutrition in one species, and a broad number of phytoplankton species is described to be able to physiologically utilize dissolved organic material and prey particles (Anderson et al. 2002, Burkholder et al. 2008, Kamjunke and Tittel 2008).

In our study, we investigated mixotrophy in a more narrow sense, focusing on heterotrophic nutrition of phytoplankton that arises from uptake of bacterial prey organisms. An increasing amount of studies points out that mixotrophy in phytoplankton seems to be rather a rule than a specialist exception (Graneli et al. 1999, Sanders and Gast 2011, Zubkov and Tarran 2008). On the other hand, the many-fold types of heterotrophic nutrition in algae (Jones 1997, Raven 1997) still keep detection and prove of mixotrophy (in the natural environment as well as in experimental studies) a methodological challenge (de Schryver et al. unpublished results). Consequently, it is almost impossible to prove for any phytoplankton species to be restricted exclusively on pure autotrophy. Nevertheless we typed five of our experimental species "autotrophs" and do not suppose any heterotrophic mode of nutrition based on DOC uptake or preying on bacteria.

This is because in diatoms, phagotrophy does not exist and our *Thallassiosira sp.* does not positively react to DOC additions in the growth medium. The latter also applies for our *Rhodomonas sp.*. *Prasinocladus marinus* does not seem to be affected by DOC additions and to our best knowledge, mixotrophy has never been described in this species. Dinoflagellates, on the other hand, are often described to rely on mixotrophy (Stoecker 1999, Davidson et al. 2012). However, if dinoflagellates are in heterotrophic mode, they frequently become conceptually part of the microzooplankton as they tend to choose bigger prey organisms than heterotrophic bacteria (Graneli et al. 1997, Stoecker et al. 1997, Stoecker 1999). Dinoflagellates are also frequently found to profit from dissolved organic material (DOM), especially from humic substances (Fagerberg et al. 2010, Gagnon et al. 2005), but there is evidence that this phytoplankton group utilizes dissolved organic nutrients bound to DOM. In our experiment, we used pure carbon (saccharose) as a DOC source and therefore excluded the latter nutritional pathway for all of our algae.

V.4 Conclusion and Outlook

To conclude, increasing concentrations of DOC resulted in increasing contribution of our mixotrophic model species to total phytoplankton biomass. At the highest added DOC concentration, *O. minima* became the dominant species in our model community. We therefore argue that mixotrophic phytoflagellates may become key players in changing planktonic ecosystems, taking advantage of their capability to graze on increasing bacterial abundances in warmer and DOC rich waters.

However, a recent study on bacterioplankton communities from the northern Baltic Sea, shows, that heterotrophic bacterial response to higher temperature, strongly depends on the nutrient regime. If nutrient levels were low, temperature had poor effect on bacterial growth (Degerman et al. 2013). Therefore, and in order to be able to properly judge the potential change in phytoplankton functional types under scenarios with increasing continental run-off, higher DOC concentrations in coastal waters and surface water warming, more studies are clearly necessary that also take into account the predicted ranges of eutrophication. On the other hand, particularly in northern Europe, freshwater tDOC often consists of brownish-coloured humic carbon, that reduces light transmission in the water column and thereby has potential to shift the ecosystem from an autotrophic, phytoplankton based to a net-heterotrophic microbial based state (Andersson et al. 2013). Since positive growth of our model species *O. minima* is light-dependent (de Schryver et al., unpublished results), this species would not necessarily profit from increased, naturally occurring DOC concentrations if these provoke shading of the water column. In contrast, mixotrophic species that depend stronger on a heterotrophic nutrition and may start to ingest particles under low light conditions, could potentially replace strict autotrophic phytoplankton taxa in humic rich waters. In order to improve our understanding of the role of tDOC in marine ecosystems, more research should therefore concentrate on experimental studies which include natural DOC components.

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DISCUSSION

Influence of dissolved organic carbon (DOC) and temperature on mixotrophic and autotrophic phytoflagellates

This study hypothesized that under nutrient-replete conditions, increasing concentrations of DOC should positively influence the growth of mixotrophic phytoflagellates, since abundances of heterotrophic bacterial prey should increase.

Results from microcosm experiments in this study on the mixotrophic chrysophyte model species *Ochromonas minima* strongly support the assumption.
In detail, it could be shown that the environmental factors DOC, nutrient availability, and warm water temperature, had positive influences on *Ochromonas minima* performance in controlled laboratory systems. Not only did DOC enhance the mixotrophs growth, but increasing concentrations of DOC also provoked a shift of the community composition of an artificial phytoplankton community from dominance of autotrophs to dominance of *Ochromonas minima.* Higher incubation water temperature had a positive influence on the relative importance of the mixotroph in the community, particularly under conditions when DOC was supplied at moderate concentrations, presumably due to a stronger positive influence of temperature on the heterotrophic, than on the autotrophic, nutrition mode of the flagellate.

Our results are well in line with findings from natural habitats, such as reports from DOC rich humic lakes on dominance of mixotrophic phytoplankton species (Jansson et al. 1996, Jones 2000). In addition, Schmidtke et al. (2006) describe very high grazing rates of *Ochromonas sp.*. in an acidic mining lake, reaching from a daily grazing rate of 68 % of the single-celled bacterial population in the hypolimnion, to 88 % in the epilimnion. Grazing on bacteria as a way to obtain particulate nutrients under limiting environmental dissolved concentrations is often described for mixotrophic phytoflagellates (Stibor and Sommer 2003, Stukel et al. 2010) and suggested to be an evolutionary strategy that allows mixotrophs to compete more successfully and to sustain viable populations under conditions not favourable for pure phototrophic species (Katechakis and Stibor 2006).
Interestingly, a time-series analysis on three sub-basins of the northern Baltic Sea revealed an increase of *Chrysochromulina sp.* with global change related increasing

surface water temperatue (Suikkanen et al. 2013). Haptophytes, such as *Chrysochromulina spp*., are supposed to host a broad number and diversity of phagotrophic species (Liu et al. 2009) and mixotrophy is confirmed for many *Chrysochromulina* species. There are, in general, very few studies that focus on the role of temperature for the physiology and performance of mixotrophic species. However, Wilken et al. 2012, previously confirmed for another mixotrophic *Ochromonas sp.* that its growth rate increases strongly with temperature, if the flagellate is grown in heterotrophic mode.

Warming sea surface temperatures, increased precipitation and continental run-off, and therefore further eutrophication and increasing terrigenous DOC concentrations in coastal areas are a result of global change, in some geographic areas, such as the northern Baltic Sea.

Results from our laboratory microcosm experiments and environmental observations on mixotrophic species, suggest a strong impact of these predicted global environmental changes on the interactions of heterotrophic bacteria, autotrophic and mixotrophic phytoplankton. Nevertheless, results from our study focus on the mixotrophic *Ochromonas minima* and cannot be generalized to all species of the whole large group of phagotrophic phytoplankton species. It is definitely necessary to conduct further similar studies on a variety of mixotrophic species that exhibit different behavioural strategies (Jones 1997), such as for example, species that do not rely essentially on photosynthesis, as *Ochromonas minima* does. Experiments on natural phytoplankton and bacteria communities should be conducted to include broader ranges of biodiversity and to study effects on species that are, within their common communities, adapted to their respective environments. Such experiments should include phytoplankton and bacterial communities from different environmental nutrient regimes, since it was found that warming and nutrient concentration interactively affect the growth of heterotrophic bacteria (Degerman et al. 2013), which should translate into effects on mixo- and autotrophic phytoplankton performance.
By working with natural communities, heterotrophic nanoflagellates (HNFs) cannot be excluded from samples. However, HNFs are, beside heterotrophic bacteria and autotrophic phytoplankton, the third major group of competitors for mixotrophic

protists, which have not been topic of this study. HNFs compete with mixotrophic flagellates for prey particles and it is therefore crucial to take HNFs into account in future, particularly, in environmental studies on the influence of DOC, temperature and nutrients for planktonic protists performance.

Clearly, the DOC sources used in our study (sacharose and milk powder) can only be seen as model substances for biologically easily degradable DOC sources. In the environment, DOC that is transported by river waters often constitutes of humic substances which gave water a brownish colour. Such compounds also shade the water column and restrict light availability for phytoplankton. Future studies should therefore take into account natural DOC substrates and further aim to discriminate effects on mixotrophic protists performance caused by light and/or DOC.

Implications for plankton food web ecology

While this study has focused on bacteria and phytoplankton as the basal producers in planktonik food webs, further studies may take into account the role of mixotrophic protists for performances of higher trophic levels.

In particular, the effects of DOC and nutrient concentrations on the dynamics of mixotrophs, autotrophs and HNFs, and the resulting influence for grazing mesozoplankton (copepods) can well be studied in mesocosm experiment designs. Experiments with freshwater plankton have shown that mixotrophic food algae can positively influence zooplankton, by providing higher amounts of nutrient per unit carbon, than pure autotrophic phytoplankton (Katechakis et al. 2005). On the other hand, one can argue, as well, that phagotrophic phytoflagellates should improve transfer efficiencies of carbon from bacteria to metazooplankton, compared to food chains that are dominated by heterotrophic nanoflagellate bacterial grazers. While pure heterotrophic organisms (HNFs) respire relatively high amounts of the ingested organic carbon, mixotrophic flagellates may compensate these losses by photosynthesis and therefore provide a higher flux of carbon to mesozooplankton. In contrast, many mixotrophic species have been described to be toxic and cause harmful agal blooms (Burkholder et al. 2008) in marine food webs. Therefore, the influence of DOC, eutrophication and rising sea surface temperatures on interactions between mixotrophic harmful species (such as some *Chrysochromulina spp.*) and heterotrophic bacteria provides further crucial topics for future studies.

Conclusions for methodology in further studies

This study yielded several implications for methodology in plankton ecology studies.

Results from this study point out the importance of DOC in phytoplankton culture. Soil extract has been used traditionally as a supplement for the growth medium for several phytoplankton species. While soil extract is an unknown mixture of different substances, providing DOC but potentially also dissolved inorganic nutrients, our results demonstrate that DOC *per se* can positively influence phagotrophic phytoplankton, if heterotrophic bacteria and sufficient dissolved nutrients are present in the culture. Though sometimes avoided in order not to fuel heterotrophic bacterial growth in phytoplankton cultures, natural sterile seawater with naturally occurring DOC, may be the better choice than artificial sea water (based on distilled water), for the culture of some mixotrophic species.

Concerning the application of catalyzed reporter deposition fluorescence in situ hybridization (CARD FISH) for the study of phagotrophy in mixotrophs, our results clarify that further development of oligonucleotide probes for targeting heterotrophic bacteria is necessary. Studying the complete grazing impact by mixotrophic flagellates on bacteria with the CARD FISH methodology is not going to be feasible as long as oligonucleotide probes match chloroplast nucleic acid sequences, which prevents discrimination of bacteria and phytoplankton.

Nevertheless, mixotrophy in phytoplankton is a widespread and globally important phenomenon (Liu et al. 2009, Stukel et al. 2010, Zubkov and Tarran 2008).

Meanwhile, global change affects marine habitats generally by increasing CO_2 partial pressures and warming sea surface waters. Large coastal marine areas in some geographic regions are confronted with increasing DOC and nutrient concentrations. All of these factors are known to influence biogeochemical carbon fluxes and to influence the dynamics of phytoplankton and planktonic heterotrophic bacteria (Hoppe et al. 2008, Kim et al. 2011, Degerman et al. 2013). The capacity of the biological carbon pump (i.e. the uptake of CO_2 by phytoplankton, its storage in phytoplankton biomass and its transport to deep water regions), and the efficiencies of marine food chains, depend to a large extend on the composition of phytoplankton functional groups. In order to judge future potential changes to the functioning of marine ecosystems and to ecosystem services, it is therefore necessary to gain a profound understanding of the roles of the different functional groups under changing environmental conditions.

The study of mixotrophic phytoplankton as a functional group works clearly at the interface of several biological and biogeochemical disciplines. For future studies, co-operation of plankton ecologists and molecular ecologists, in particular, is of crucial importance for development of powerful methodological tools.

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Contribution of Authors

CHAPTER 1: CARD-FISH AS A MOLECULAR METHOD TO VISUALIZE PHYTOPLANKTON ASSOCIATED BACTERIA - IS IT APPROPRIATE FOR DETECTION AND QUANTIFICATION OF MIXOTROPHY?

Vera de Schryver (VdS), Miguel Angel Ballen Segura (MABS) and Kevin Nahélou (KN)

Planning of laboratory experiments: VdS, MABS
Conduction of experiments, analysis of samples: VdS, MABS, KN
Data analysis and writing: VdS

CHAPTER 2: COMPOSITION AND CONCENTRATION OF DISSOLVED ORGANIC CARBON INTERACTIVELY AFFECT MIXOTROPHIC *OCHROMONAS MINIMA* POPULATION YIELD BY ALTERING SUPPLY WITH BACTERIAL PREY

Vera de Schryver (VdS), Jennifer Schoenn (JS), Christophe Lambert (CL), Herwig Stibor (HS)

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Design of experiment: VdS, HS
Planning and conduction of experiment: VdS, JS, CL
Data analysis and writing: VdS with assistance of HS

CHAPTER 3: INFLUENCE OF DISSOLVED ORGANIC CARBON ON GROWTH AND COMMUNITY COMPOSITION OF PHYTOPLANKTON ASSOCIATED WITH BACTERIA

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Mixotrophie et dynamique de l'écosystème pelagique

Résumé
Les espèces protistes ont été traditionnellement classifiées comme des plantes or des animaux en raison de l'absence ou présence des chloroplastes. L'état actuel de la connaissance indique qu'un grand nombre d'espèces protistes portent des chloroplastes mais que physiologiquement elles sont capables d'utiliser l'autotrophie (photosynthèse) ou l'hétérotrophie pour se nourrir. La combinaison de ces deux modes trophiques par une même cellule est nommée mixotrophie. Chez les protistes l'hétérotrophie peut s'effectuer soit par la consommation des particules par phagocytose, e.g. des proies bactériennes, ou bien par l'absorption des composants organiques dissouts, i.e. osmotrophie.
La mixotrophie est de plus en plus décrit chez les protistes dans tous les habitats aquatiques. Les écologistes du plancton constatent la récurrence de la mixotrophie chez les formes traditionnelles « phyto »plancton et micro »zoo »plancton. Cependant, identifier et quantifier la mixotrophie reste toujours un défie méthodologique. Dans cette étude nous nous sommes intéressés à la mixotrophie chez les espèces phytoplanctoniques marines, en particulier à leur nutrition phagotrophique de proies bacteriennes.

Nous avons testés des techniques modernes afin d'identifier la mixotrophie dans des cellules phytoplanctoniques. La technique cytogénétique d'hybridation in situ Card-FISH en utilisant de sondes d'ARN ribosomique 16S a été effectuée suivant des protocoles existant pour des bactéries et des protistes. Cette technique s'est avérée être un outil précieux pour visualiser des groupes phylogénétiques bactériens en association avec le phytoplancton à l'aide de la microscopie à épifluorescence, sans avoir besoin d'un isolement préalable des cellules ou des interférences avec l'association microbienne. Cependant, la méthode a échoué pour visualiser mixotrophie chez le phytoplancton car la sonde eubactérienne générale (EUB338) combine une large gamme d'espèces phytoplanctoniques, ce qui rend impossible de discriminer les signaux fluorescents provenant de tissus bactérienne ou phytoplanctonique.

Le contexte de ces études est le phytoplancton et les bactéries hétérotrophes lesquels constituent des principaux concurrents pour les nutriments inorganiques dissouts. Dans le cas où la croissance bactérienne est limitée par le carbone, l'augmentation de la concentration de carbone organique dissous (DOC) renforce la croissance bactérienne et la consommation de nutriments dissous et ainsi affecte négativement la croissance du phytoplancton autotrophe. Cependant, les consommateurs de bactéries, i.e. phytoflagellés mixotrophes, peuvent être favorisés dans de telles situations car la hausse de DOC donne lieu à l'abondance plus élevé des proies bactériennes.

En outre, nos résultats indiquent un potentiel effet positif de la température sur le mode de nutrition hétérotrophe de l'espèce, ainsi qu'une croissante contribution des espèces mixotrophes au sein des communautés de phytoplancton dans des conditions des hautes températures des eaux de surface de la mer.

Mots clés : espèces protistes, mixotrophie, écosystème, pélagique, Dynamique

Mixotrophy and pelagic ecosystem dynamics

Abstract

Protist species were traditionally classified morphologically as either „plants“ or „animals“, based on the absence or presence of chloroplasts. State of science is that a high number of protist species carry chloroplasts but are nutritionally able to employ both autotrophy (photosynthesis) and heterotrophy within a single cell. This combination of autotrophic and heterotrophic mode of nutrition within a single species is named mixotrophy. In protists, heterotrophy can be realized either by the uptake of food particles (e.g. bacterial prey) through phagocytosis or by the uptake of dissolved organic compounds (i.e. osmotrophy).

Mixotrophy is globally and increasingly described in protists from all types of aquatic habitats. Plankton ecologists nowadays assess mixotrophy among the traditionally typified “phyto”plankton and mikro”zoo”plankton species as regularity. Nevertheless, detection and quantification of mixotrophy is still a methodological challenge. In this study, we focused on mixotrophy in marine phytoplankton species and put emphasis on its phagotrophic nutrition from heterotrophic bacterial prey.

State of the art methodology was tested to visualize mixotrophy in single phytoplankton cells. Catalyzed reported deposition-fluorescence in situ hybridization (Card-FISH), using 16S ribosomal RNA probes, was employed based on existing protocols for bacteria and protists. The method proved to be a valuable tool to visualise bacterial phylogenetic groups in association with phytoplankton by epifluorescence microscopy without need for prior isolation of cells or interference with the microbial association. However, the method failed to visualize mixotrophy in phytoplankton since the general eubacterial probe (EUB338) hybridised a broad range of phytoplankton species making it impossible to discriminate fluorescent signals originating from bacterial or phytoplankton tissue.

Background of these studies is phytoplankton and heterotrophic bacteria being major competitors for dissolved inorganic nutrients. In case that bacterial growth is carbon limited, increasing concentrations of degradable dissolved organic carbon (DOC) enhance bacterial growth and consumption of dissolved nutrients and thereby negatively affect autotrophic phytoplankton growth. Bacteria consuming mixotrophic phytoflagellates, however, may gain in importance in such situations since DOC provokes higher bacterial prey supply.

In addition, our results indicate a potential positive effect of temperature on O. minima´s heterotrophic nutrition mode, and indicate a potential increasing contribution of mixotrophic species to phytoplankton communities under increasing sea surface water temperatures.

Keywords : Protist species, mixotrophy, ecosystem, pelagic, Dynamic