Influence of nutrients and light on autotrophic, mixotrophic and heterotrophic freshwater chrysophytes

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ABSTRACT: Chrysophyte algae show wide variation in their nutritional modes, which can be especially advantageous in oligotrophic conditions. However, the capacities and strategies of the algae to adapt to changing conditions may vary due to different nutritional requirements of individual species. In this context, comparative analyses evaluating the physiological range of mixotrophic chrysophytes are important to predict possible changes in phytoplankton composition. We compared 4 freshwater chrysophytes — *Poteriospumella lacustris*, *Poterioochromonas malhamensis*, *Dinobryon divergens* and *Mallomonas annulata* — under different growth conditions. The diatom *Phaeodactylum tricornutum* served as a photoautotrophic reference strain. We demonstrate active growth of *P. lacustris* and the mixotrophic *P. malhamensis* under chemoheterotrophic conditions. Neither of the chrysophytes were growing photoautotrophically; however, *P. malhamensis* showed some photosynthetic activity and survived longer when kept in the light. *M. annulata*, *P. tricornutum* and the mixotrophic *D. divergens* showed light-limited growth when kept in inorganic media. Even though *D. divergens* consumed living bacteria, it did not grow in the dark in the presence of the bacteria only. We detected large differences in the general nutritional preferences between the 2 mixotrophs in relation to the nutritional features of all investigated strains.

KEY WORDS: Mixotrophy · Oxygen evolution · Carbon · Culturing conditions · *Phaeodactylum tricornutum*

INTRODUCTION

Mixotrophic algae are able to meet their carbon demands by assimilating inorganic carbon (CO_2) as well as by taking up organic molecules. The uptake of organic substances is not limited to dissolved molecules (osmotrophy) but can also be accomplished by ingestion of detritus or living bacteria (phagotrophy). Chrysophytes (throughout the manuscript, this term refers to the chrysophytes *sensu lato*, comprising the classes Chrysophyceae and Synurophyceae [Andersen et al. 1999, Kristiansen & Preisig 2007, Jordan & Iwataki 2012]) are of special interest because their abundance may increase during phases of lake reoligotrophication (Gaedke 1998, Jeppesen et al. 2005)

and because they often dominate mixotrophic phytoplankton populations (De Hoyos et al. 1998, Watson et al. 2001, Kamjunke et al. 2007). Chrysophytes are geographically widespread and abundant in freshwater and marine habitats as well as in soils (Sandgren 1988, Boenigk & Arndt 2002, Massana 2011). While a large number of chrysophyte genera show mixotrophic traits (Porter 1988, Sanders & Porter 1988), all the different metabolic lifestyles ranging from exclusively chemoheterotrophic to exclusively photoautotrophic nutrition can be found (Raven et al. 1995).

Several parameters may affect the growth competitiveness of mixotrophic chrysophytes. Besides carbon and phosphorous availability (Rothhaupt 1996a), light (de Castro et al. 2009), pH (Moser & Weisse

2011b) and temperature (Moser & Weisse 2011a, Wilken et al. 2013) have also been shown to be important factors in laboratory studies. Furthermore, some chrysophytes require vitamin supply (Heinrich 1955). Mixotrophic chrysophytes are thought to be very competitive in oligotrophic waters because some of them may ingest bacteria, which are utilized as carbon and phosphorus source (Thingstad et al. 1996, Kamjunke et al. 2007, Unrein et al. 2007). Still, each of the mentioned parameters is likely to affect each chrysophyte species in a different way (Watson & McCauley 2010, Moser & Weisse 2011b), making it difficult to predict the success of certain mixotrophic species under changing conditions.

In the present study, we compare nutritionally different mixotrophic chrysophyte species to each other as well as to purely photoautotrophic or purely chemoheterotrophic reference species. We aimed to demonstrate how different chrysophytes are affected by nutritional parameters under identical conditions and by this to understand whether the observed differences may influence the competitive fitness of a single species. To this end, we tested different culturing conditions in order to find the most suitable conditions for physiological comparisons. Further, we specified which component of the culturing media limited maximum cell numbers of each protist to make sure that the macronutrients and not the trace metals or vitamins were the limiting factor. We performed comparative studies examining the influence of light and different carbon sources on all strains. Additionally, we compared photosynthetic capacities of all strains by conducting oxygen evolution measurements to complement results from the growth experiments.

MATERIALS AND METHODS

Strains and standard culturing conditions

The chrysophytes investigated in the present study were isolated from oligotrophic, alpine or pre-alpine freshwaters (Table 1) and include 2 mixotrophic strains, *Dinobryon divergens* FU18K_A (xenic) and *Poterioochromonas malhamensis* DS (axenic). The colourless heterotrophic *Poteriospumella lacustris* (strain JBM10) (axenic) and the photoautotrophic *Mallomonas annulata* WA 18K_M (xenic) were chosen as reference strains. As a photoautotrophic reference strain, we also studied the diatom *Phaeodactylum tricornutum* UTEX646.

Poteriospumella lacustris was cultured in modified inorganic basal medium (pH 7.2) (Hahn 2003) without NiCl$_2$ and with addition of dissolved organic matter (DOM) sources of 1 g l^{-1} peptone (Roth), 1 g l^{-1} yeast extract (Roth) and 1 g l^{-1} α-D-glucose. *Dinobryon divergens* and *Mallomonas annulata* were grown in liquid WC medium (pH 8) (Guillard & Lorenzen 1972) with modified concentrations of TES buffer (C$_6$H$_{15}$NO$_6$S 500 µM), NaHCO$_3$ (375 µM), K$_2$HPO$_4$ (32 µM), NaNO$_3$ (500 µM) and MnCl$_2$ (0.09 µM). The standard medium for *Poterioochromonas malhamensis* was modified bacillariophycean medium (BM) (pH 7.2) (Schlösser 1994) with 236 µM Na$_2$CO$_3$, 27 µM iron(III) citrate hydrate, 214 µM citric acid, 60 nM MnSO$_4$, 200 µg l^{-1} thiamine-HCl, 10 µg l^{-1} biotin and DOM. *Phaeodactylum tricornutum* was grown in modified f/2 medium (pH 7) (Guillard & Ryther 1962, Guillard 1975) with artificial half-concentrated sea salts (16.6 g l^{-1} Tropic Marin®, Dr. Biener) and 0.09 µM manganese or on solid plates of modified f/2 (with 1.5% agar) for culture maintenance. The axenic state of the cultures was monitored regularly by light and fluorescence microscopy, using an automatic particle counter and by screening culture aliquots for growth of bacterial colonies on solid media (1.5% agar) containing DOM. A consortium of several bacterial strains served as prey during feeding experiments. The bacteria were isolated from *Dinobryon divergens* cultures by plating a culture sample on WC medium including DOM and incubating the plate for 1 wk at room temperature. The bacterial consortium was transferred to fresh plates monthly without further purification. The bacteria were grown in liquid WC medium with DOM for feeding experiments.

Cultures were generally grown at 20°C in Erlenmeyer flasks without shaking under sterile conditions and at a light intensity of 30 to 40 µmol photons m^{-2} s^{-1} on the surface of the culture flasks. If not noted otherwise, the light:dark cycle was 16 h:8 h.

Cell counts of protists

Cell counts and the determination of the biovolume of protists were performed with an automatic particle counter according to the Coulter principle ('Coulter counter', Multisizer III, Beckman Coulter) using a capillary with an aperture of 100 µm diameter. Directly prior to measurements, 100 to 900 µl of a cell suspension were diluted in 12 ml of the electrolyte IsotonII-Diluent (Beckman Coulter) by pipetting. Measurements were performed in triplicates for each sample and averaged. To reduce background signals from dust, precipitates and fibers, a sample of the sterile control was analyzed and subtracted. To gain

Table 1. Algal strains investigated in the present study

Algal strain and origin	Axenic ?	Reference for strain description	Carbon acquisition	Habitus	References for phagotrophy
Mallomonas annulata WA 18K_M Lake Wallersee, Austria	No	J. Boenigk (unpubl.)	Autotroph	Unicellular	None
Dinobryon divergens FU 18K-A Lake Fuschelsee, Austria	No	J. Boenigk (unpubl.)	Mixotroph	Colonial	Jones & Rees (1994), Veen (1991)
Poterioochromonas malhamensis DS Lake Constance, Germany	Yes	Hahn & Höfle (1998)	Mixotroph	Unicellular, stalks	Hahn & Höfle (1998)
Poteriospumella lacustris JBM10 Lake Mondsee, Austria	Yes	Findenig et al. (2010)	Heterotroph	Unicellular	Boenigk et al. (2006)
Phaeodactylum tricornutum Bohlin UTEX 646 Baltic Sea, Finland	No	Droop (1954), De Martino et al. (2007)	Autotroph	Unicellular	None

the cell density (i.e. cell number per milliliter), the curve area corresponding to the algal cells was calculated. The curve area limits were 3 to 20 µm for *Poteriospumella lacustris*, *Phaeodactylum tricornutum* and *Poterioochromonas malhamensis*, 3.5 to 20 µm for *Dinobryon divergens* and 4 to 20 µm for *Mallomonas annulata*. Cell volume and cell diameters were both extracted from the same size distribution graphs after measurements with the Multisizer III by choosing 'diameter' or 'volume' as the *x*-axis.

Except for *Dinobryon divergens*, dead cells or cell fragments were clearly distinguishable from living cells by size. To obtain a better evaluation of the number of living cells of *D. divergens*, we subtracted 30% of the cell counts representing empty loricas during exponential phases in those cases where numbers of living cells were required, but this subtraction was not used for the determination of growth rates (for details, see Rottberger et al. 2013).

Cell counts of bacteria

Bacterial cell counting was conducted with unstained cells using a phase contrast microscope and a micrometer ocular at 640× total magnification. Cell counts of each sample were performed in quadruples and averaged. The concentration of bacteria added during feeding experiments was adjusted to calculated optical densities (ODs) of 0.01 and 0.005. This was achieved by measuring the OD at $\lambda = 600$ nm of bacterial stock solutions with an OD_{600} between 0.1 and 0.5 using a spectrophotometer and by calculating the necessary volume of stock suspension to achieve final ODs of 0.01 and 0.005 in the culturing flask. The OD measurements were exemplarily cor-

related to microscopic cell counts of living bacteria grown in WC medium with DOM by a dilution series ranging from OD 0 to 0.5. Bacterial cell numbers were then calculated by the formula of the linear regression curve over all data points (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a071 p179_supp.pdf).

Accompanying bacteria in the cultures of *Dinobryon divergens* and *Mallomonas annulata* were counted exemplarily using phase contrast microscopy in mid-exponential phase at a density of 47 000 cells ml^{-1} of *Dinobryon divergens* and 73 000 cells ml^{-1} of *Mallomonas annulata*. Cultures of *D. divergens* and *M. annulata* were grown under standard conditions in triplicates with an initial inoculum of 1000 algal cells ml^{-1}. Each triplicate was sampled, 4 bacterial counts per sample were conducted, and all 12 counts per alga were averaged.

Growth experiments

Growth experiments were performed in triplicates. Protist cultures were inoculated with a start abundance of 1000 to 10 000 cells ml^{-1}. Cultures were grown in the light (16 h light:8 h dark) up to a cell density of approximately 50 000 to 100 000 cells ml^{-1} during light-limitation experiments prior to exposing them to a 4 h light:20 h dark (6 h light:20 h dark for *Mallomonas annulata*) or 8 h light:16 h dark cycle. For experiments in complete darkness, culture flasks were wrapped with aluminium foil directly after inoculation.

In aerated treatments, dry, double sterile-filtered air (0.2 µm PTFE-filters, Whatman) was bubbled into the culture flasks through silicon plugs. In agitated

treatments, culture flasks were placed on a horizontal shaker (GFL 3020, 100 rpm).

To test photoautotrophic growth, all protists were grown under standard conditions and in their standard medium (see 'Strains and standard culturing conditions' above), except that DOM was omitted in the culturing media of *Poteriospumella lacustris* and *Poterioochromonas malhamensis*. DOM was not added to the culturing media of *P. lacustris* or *P. malhamensis* during feeding experiments. Feeding experiments were performed with either autoclaved or living bacteria that had been washed twice with 0.9% sodium chloride. Autoclaved bacteria were added to the culturing media of all protists to a calculated bacterial OD_{600} of 0.01 in the culture (see previous section). In a separate experiment, living bacteria were added to the culturing medium of *Dinobryon divergens*, to a calculated bacterial OD_{600} of 0.005. Bacteria were added to each culture flask prior to inoculation with the protists. Growth of the protists was analysed by automatic particle counting.

Fluorescence labelling of bacteria was used as a tool to visualize the ingestion of undamaged bacteria (Sherr et al. 1987, Chrzanowski & Simek 1990) and was accordingly applied to confirm active ingestion of non-autoclaved bacteria by *Dinobryon divergens*. A stock solution of fluorescently labelled living bacteria was generated by staining cells with SYTO®9 Green fluorescent nucleic acid stain (Invitrogen) in a small volume of medium (100 µl) for 1 h. After that the solution was washed to remove surplus dye. From the washed stock solution, fluorescently labelled bacteria were added to *D. divergens* cultures in exponential growth phase with an OD_{600} = 0.005. The volume of stained bacteria needed to achieve this OD was determined by measuring the OD of a parallel, non-stained bacterial culture that was treated identically, except that medium was used instead of staining solution. Ingestion of bacteria was analysed after 2 to 6 h using a fluorescence microscope to gain an overview and a confocal laser scanning microscope for more detailed studies of single protist cells.

Maximum yield limitation experiments

Potential limitations on the maximum cell numbers of each investigated strain were tested under standard conditions by increasing the concentration of DOM, glucose, the nitrate source, the phosphate source, the vitamin solution or the trace metal solution during stationary phase to 3-fold the standard concentrations.

Oxygen measurements

Cells were harvested during late exponential growth phase by gentle centrifugation ($<1500 \times g$). Cell densities were adjusted to $>10^6$ cells ml^{-1} using a 1:1 mixture of culture supernatant and fresh culturing medium. Oxygen measurements were performed at 20°C with 1 ml of the cell suspensions in a Clark-type electrode (Hansatech). Samples were acclimatized to the respective light intensity (0 to 1000 µmol photons m^{-2} s^{-1}) within the electrode chamber for 10 min without stirring. Measurements were then performed within 4 min of continuous illumination and gentle stirring (30 rpm). The biovolume of the cells in the samples was determined after oxygen measurements using the Coulter counter, and rates were then calculated to fmol O$_2$ µm^{-3} h^{-1}. All oxygen evolution rates were determined from triplicates measured within 3 successive days.

Statistics

The significance of differences was calculated by a Student's *t*-test using SigmaPlot 12.0 (Systat Software). Here, normality distribution was tested by a Shapiro-Wilks test with a confidence interval of 95%, a score of 0.05 and a p-value to reject normality of 0.05.

RESULTS AND DISCUSSION

Selection and characterization of strains

For comparative physiological studies under laboratory conditions, we chose 2 mixotrophic freshwater species of the genera *Poterioochromonas* and *Dinobryon* that are both well-described in the literature (e.g. Bird & Kalff 1987, Caron et al. 1990, Boenigk et al. 2006, Watson & McCauley 2010) and compared them to one photoautotrophic (*Mallomonas annulata*) and one heterotrophic chrysophyte (*Poteriospumella lacustris*). In order to compare the results to a physiologically and genetically well-investigated unicellular organism that also belongs to the stramenopiles, we studied the brackish-water diatom *Phaeodactylum tricornutum*.

Bacteria were not detected in standard cultures of *Phaeodactylum tricornutum* but occasionally appeared when the cells were grown in media containing DOM. *Dinobryon divergens* and *Mallomonas annulata* were uni-algal but not bacteria-free in all

media utilized for the experiments. Cultivation of bacteria-free *Dinobryon sertularia* has already been described (Lehman 1976); however, our attempts to purify *D. divergens* via dilution, by a single-cell pipetting approach or by incubation with different antibiotics (mainly of the β-lactame group) were unsuccessful. It has been suggested that for *D. cylindricum*, the presence of bacteria or organic compounds might be obligatory due to certain unknown growth factors (Caron et al. 1993).

Optimization of culture conditions

Cultures of different chrysophytes have successfully been maintained in batch cultures without agitation (Caron et al. 1993), with occasional agitation (Rothhaupt 1996b), with continuous agitation (Van Donk et al. 2009), with air-bubbling (Maberly et al. 2009) or with supply of 4% CO_2 (Myers & Graham 1956). In the present study, we tested the conditions of no agitation, shaking and aeration (Fig. 1). Shaking resulted in slower growth of *Mallomonas annulata*, *Dinobryon divergens* and *Poterioochromonas malhamensis* (growth rates were reduced by 55.8, 32.2 and 54.2%, respectively) than without agitation, while *Poteriospumella lacustris* and *Phaeodactylum tricornutum* performed equally at all conditions. As the CO_2 supply might be limiting in non-agitated culture flasks, we grew aerated cultures in parallel

and found higher growth rates for *D. divergens* (means ± SD: μ = 0.39 ± 0.01 d^{-1} compared to μ = 0.23 ± 0.01 d^{-1} in the agitated treatment), while the growth of *M. annulata* was lower (μ = 0.50 ± 0.02 d^{-1} compared to μ = 0.57 ± 0.01 d^{-1}) (Fig. 1). Growth rates of *P. tricornutum* were nearly identical (μ = 1.03 ± 0.05 d^{-1} compared to μ = 1.04 ± 0.03 d^{-1}). Aeration enhanced growth of *M. annulata* and *P. tricornutum* in terms of maximum cell densities (by 29.2 and 59.1%, respectively). In contrast to *P. tricornutum* (Johnston & Raven 1996, Matsuda et al. 2011), chrysophytes including *Mallomonas* species apparently lack effective carbon-concentrating mechanisms (CCM) (Maberly et al. 2009, Bhatti & Colman 2011). Consequently, the investigated chrysophytes may be more affected by the slow diffusive CO_2 supply in non-agitated cultures than *P. tricornutum.* As demonstrated earlier (Herth & Zugenmaier 1979, Veen 1991), *Dinobryon divergens* is very sensitive to changes in the experimental set-up (e.g. sensitive to mechanical stimuli or to changes in light regime or temperature). Accordingly, under aerated conditions, we found colony fragmentation, which increased the number of unicellular individuals (Fig. S2 in the Supplement). However, we did not observe any impact on cellular motility and shape of the loricas under aeration, while the growth rates of *D. divergens* were increased. Taken together, culturing all strains without agitation was the most convenient unified condition for comparison.
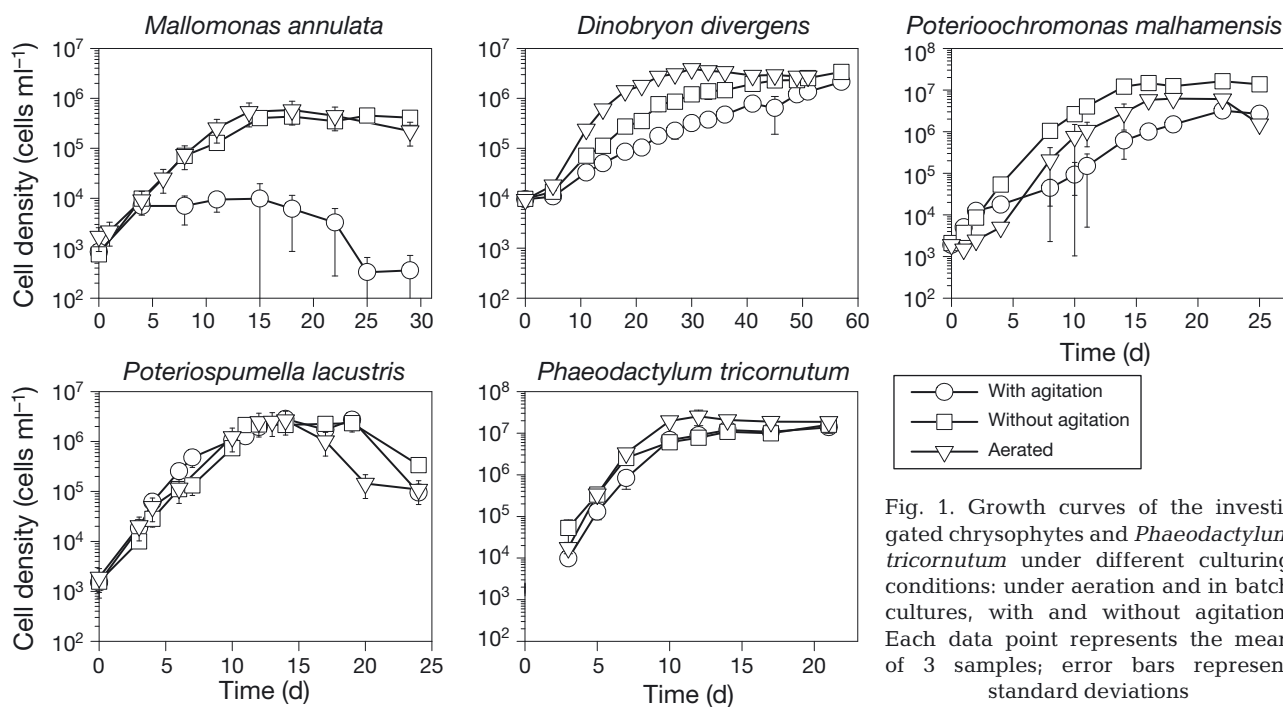

Fig. 1. Growth curves of the investigated chrysophytes and *Phaeodactylum tricornutum* under different culturing conditions: under aeration and in batch cultures, with and without agitation. Each data point represents the mean of 3 samples; error bars represent standard deviations

Influence of bacteria

Under photoautotrophic conditions, sustained growth of *Phaeodactylum tricornutum*, *Dinobryon divergens* and *Mallomonas annulata* was observed, while *Poterio-ochromonas malhamensis* and *Poteriospumella lacustris* failed to grow (Fig. 2; light, no bacteria).

Feeding with autoclaved bacteria increased the growth of *Poterioochromonas malhamensis* and *Poteriospumella lacustris* but not that of *Dinobryon divergens* and *Phaeodactylum tricornutum* (t-test, p = 0.635 and p = 0.387) (Fig. 2). *Mallomonas annulata* even showed slower growth rates in the presence of auto-claved bacteria (means ± SD: μ = 0.57 ± 0.01 reduced to μ = 0.11 ± 0.06 when autoclaved bacteria were added). Obviously, the autoclaved bacteria were not exploited as a major source of energy and carbon in the light or dark by *M. annulata*, *P. tricornutum* or *D. divergens*. However, addition of living bacteria to cultures of *D. divergens* resulted in a slight and significant (t-test, p = 0.001) increase of growth rates (μ = 0.29 ± 0.01 d^{-1}) compared to non-supplied cultures (μ = 0.25 ± 0.01 d^{-1}) (Fig. S3 in the Supplement). Fluorescently labelled living bacteria were clearly visible inside of *Dinobryon* cells, demonstrating ingestion of bacteria by the alga (Fig. 3, Fig. S4 in the Supplement).

In accordance with other studies on *Dinobryon* spp. (Caron et al. 1993, Kamjunke et al. 2007), the increase of growth rates obtained by additional
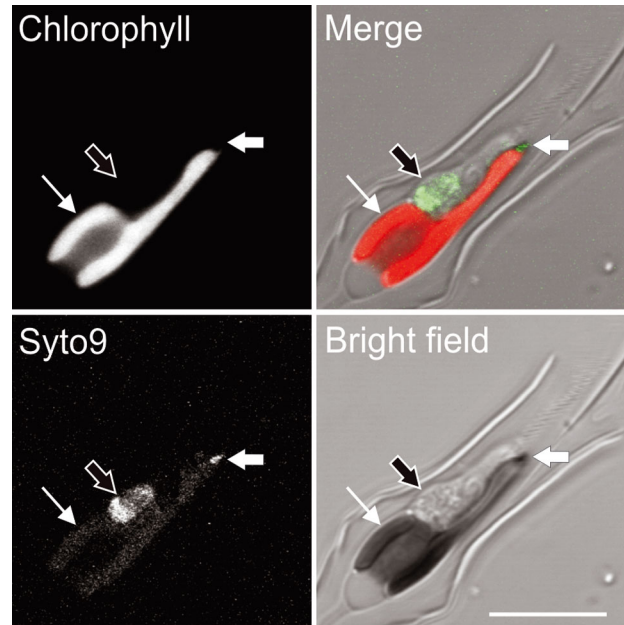

Fig. 3. Micrographs of *Dinobryon divergens* fed with living fluorescently labelled bacteria. Bacteria were labelled with a fluorescent dye (Syto®9). Ingested bacteria were visible by green fluorescence inside of the algal cell (black arrow). Additionally, the eyespot of *D. divergens* showed green autofluorescence (thick white arrow), while the plastid showed red autofluorescence (thin white arrow). A slight background fluorescence of the chloroplast was visible in the green fluorescence channel (Syto®9; see Fig. S4 in the Supplement for additional controls). Scale bar = 10 μm, applies to all micrographs
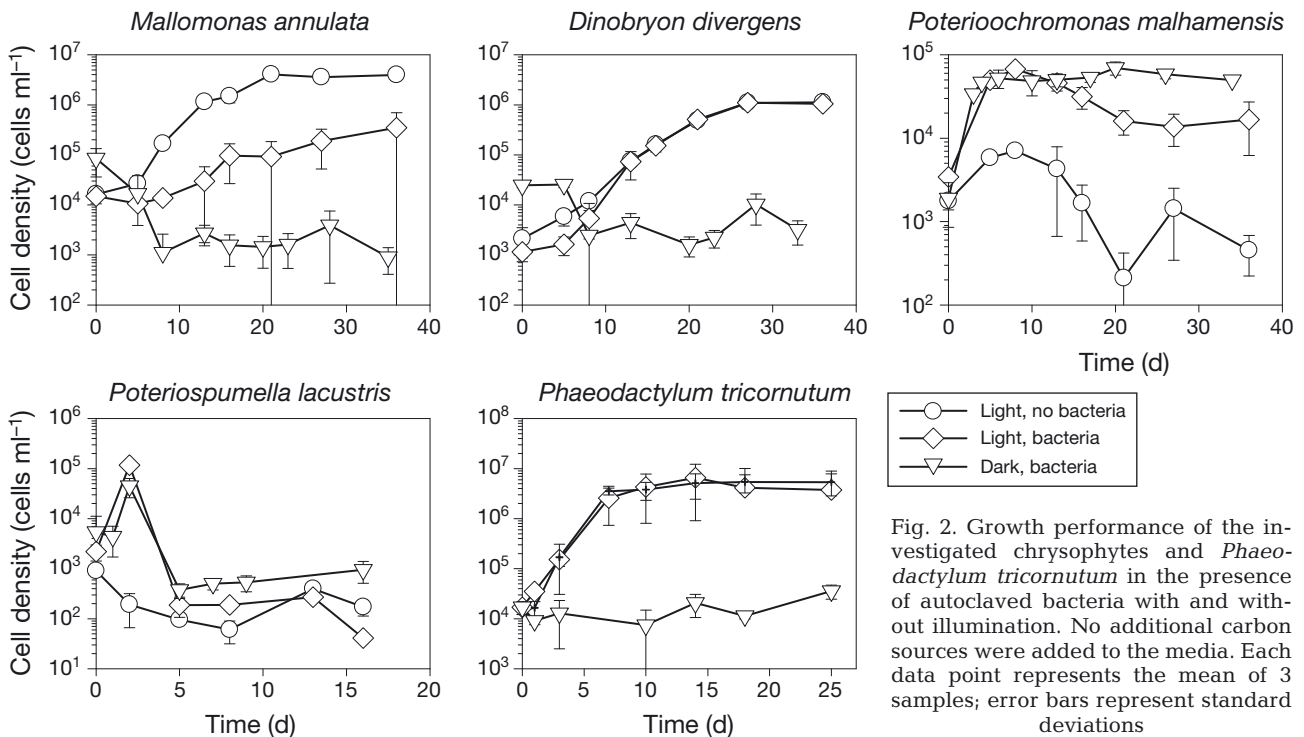

Fig. 2. Growth performance of the investigated chrysophytes and *Phaeodactylum tricornutum* in the presence of autoclaved bacteria with and without illumination. No additional carbon sources were added to the media. Each data point represents the mean of 3 samples; error bars represent standard deviations

supply with living bacteria was lower than the increase obtained by additional CO_2 supply via aeration, which indicates a stronger limitation by photosynthesis than by organic nutrient sources under the conditions applied. The outcome of feeding experiments with autoclaved bacteria may have been affected by the accompanying bacteria (see 'Selection and characterization of strains' above) in cultures of *Dinobryon divergens* and *Mallomonas annulata*. Bacterial densities under standard conditions were 10^7 cells ml^{-1} in cultures of *M. annulata* and 5×10^6 cells ml^{-1} in cultures of *D. divergens* in mid-exponential phase. Due to feeding on dead algal material, more bacteria were present in later growth phases. Less than 1 ml of exponentially growing algal cultures per 50 ml was used for inoculation. Thus, at the beginning of growth experiments, the maximum bacterial densities were 1×10^5 cells ml^{-1} in cultures of *D. divergens* and 2×10^5 cells ml^{-1} in cultures of *M. annulata*. For the feeding experiments, 3×10^6 cells of autoclaved bacteria were added; hence, there were ~15-fold more dead than living bacteria present at the beginning. The dead bacteria provided a source of dissolving organic material for the accompanying bacteria, which probably grew much faster in these experiments than after the addition of living bacteria or no bacteria. The growing bacteria perhaps depleted some limiting resources quickly (e.g. nitrate), so that *M. annulata* grew slower in experiments with autoclaved bacteria (Fig. 2). Apparently *D. divergens* could not profit from the increase in living bacteria because ingestion rates were already maximal at standard conditions. However, the cause for an increase of growth rates of *Dinobryon divergens* in feeding experiments with living bacteria remains unclear. A possible explanation would be that a portion of the bacteria might not have been accessible for *D. divergens* simply because bacterial cell agglomerates were too large to be taken up (Fig. S5 in the Supplement). Using different fluorescent beads, size selection was observed for *Dinobryon* spp. in field studies (Bird & Kalff 1987) and in laboratory experiments (Veen 1991). However, *Poterioochromonas malhamensis* and *Poteriospumella lacustris* apparently were able to exploit the cell agglomerates in our study because both showed growth in the presence of the autoclaved bacteria (Fig. 2). Accordingly, *P. malhamensis* cells have been found to graze on organisms 2- to 3-fold larger than their own cell diameter (Zhang & Watanabe 2001).

Nutritional limitations in the stationary phase

To identify further limiting factors for the maximum cell yield, we examined the role of macronutrients (organic C, N and P) or supplements (vitamins and trace metals) (Fig. 4). Nitrate was the most limiting nutrient for the photoautotrophic *Mallomonas annulata* as cell densities increased by 68 % after addition of 1 mM nitrate during the stationary phase. Nitrate and phosphate concentrations may also influence the growth response of *Mallomonas* species in natural habitats (Scott et al. 2009) and under laboratory conditions (Kim et al. 2009, Lee et al. 2012). Kim et al. (2009) showed that different *Mallomonas caudata* strains had different growth rates at identical nitrate concentrations, suggesting that nitrate turnover might be a key element of growth response in *Mallomonas* spp. Maximum cell densities of *M. caudata* varied around 10^4 cells ml^{-1} (Kim et al. 2009), whereas we observed up to 5×10^5 cells ml^{-1} for *M. annulata*, indicating that enhanced biomass generation in unialgal laboratory cultures of *Mallomonas* spp. may be accomplished by excessive addition of nitrate to the respective media. However, this hypothesis requires further investigation since the bacteria in cultures of *M. caudata* and *M. annulata* are additional nitrate consumers, which introduce a possible source of variation in bacterial cell density and nitrate demand between the cultures.

The growth of the heterotrophic *Poteriospumella lacustris* and the mixotrophic *Poterioochromonas malhamensis* was limited by the organic matter (peptone, yeast extract and glucose) (Fig. 4). As peptone and yeast extract are an undefined mixture of organic compounds, this finding alone is difficult to interpret. However, we could show that organic carbon is one of the limiting compounds in the dissolved organic matter by adding exclusively glucose to stationary phase cultures, which led to a 2.5-fold increase in maximum cell densities of *P. malhamensis*. Cell densities of *P. lacustris* were stable after the addition of glucose, while cell densities decreased rapidly in the control culture without added glucose.

None of the separately tested components (nitrate, phosphate, vitamin solution or trace metal solution) caused a positive growth response in the stationary phase of *Dinobryon divergens* and *Phaeodactylum tricornutum* cultures. Nonetheless, it is possible that *P. tricornutum* as well as *D. divergens* were limited by a combination of several elements (e.g. PO_4^{3-} and NO_3^-) or by other factors not tested (e.g. optimal Ca:Mg ratios; Lehman 1976). In fact, a combined depletion of phosphate and nitrate has recently been
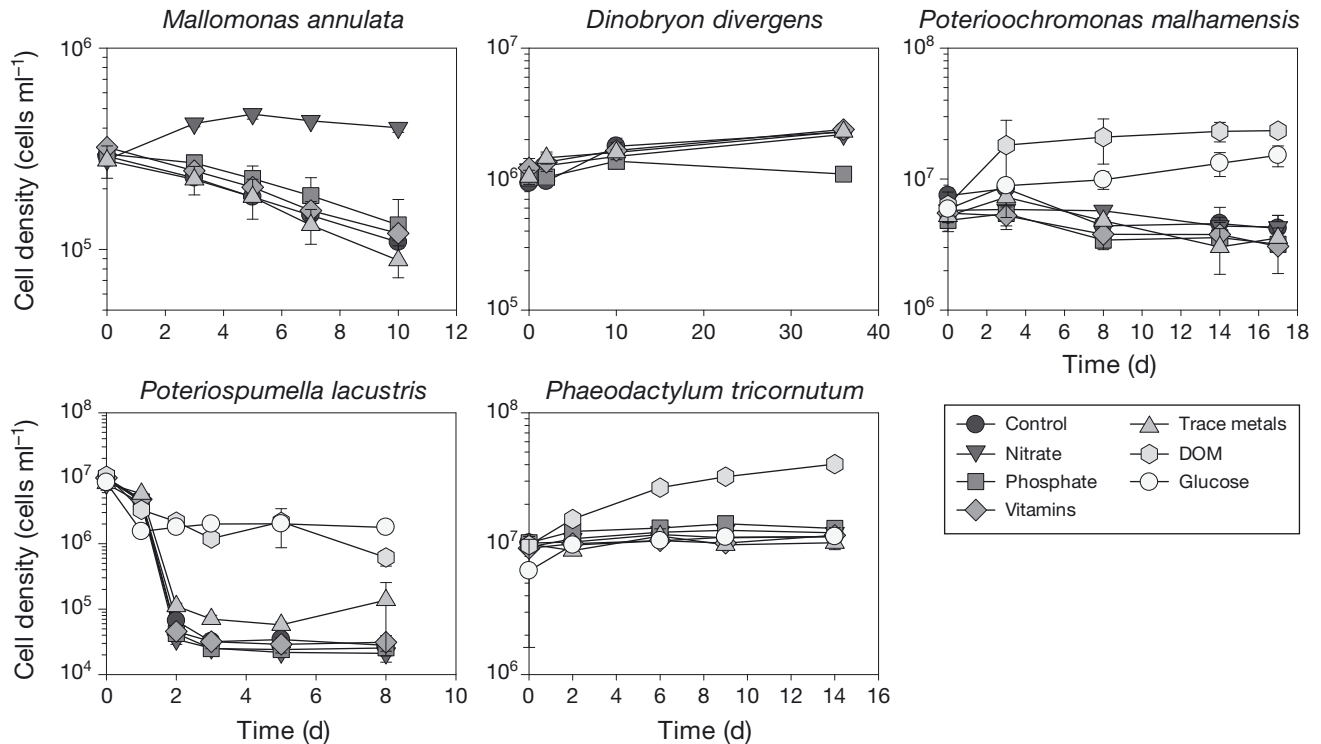
Fig. 4. Response of the investigated chrysophytes and *Phaeodactylum tricornutum* to the addition of nutrients in the stationary phase. Growth curves of protists after addition of 2× concentrated (for individual concentrations, see 'Strains and standard culturing conditions') nitrate source, phosphate source, vitamin solution, trace metal solution, glucose or DOM in stationary phase (time point 0). The control, also in stationary phase, was supplemented with 1 ml of sterile double-distilled water. Each data point represents the mean of 3 samples; error bars represent standard deviations

shown at the onset of the stationary phase for *P. tricornutum* grown in aerated bioreactors with similar growth rates ($\mu = 1.05$, deduced from cell densities shown in Fig. 1 by Valenzuela et al. 2012) compared to our aeration experiments ($\mu = 1.04 \pm 0.03$; our Fig. 1). Addition of potassium phosphate to *D. divergens* resulted in a slight decline of particle numbers (Fig. 4) and a strong decline of vital cells according to a microscopic survey (Fig. S6 in the Supplement). Potassium toxicity would explain the death of the alga but is discussed controversially in the literature (Lehman 1976, van Donk et al. 1988).

Influence of dissolved organic matter

In contrast to experiments under photoautotrophic conditions, *Poteriospumella lacustris* and *Poterioochromonas malhamensis* grew well osmotrophically when DOM was supplied reaching maximum cell densities of $>5 \times 10^6$ cells ml^{-1}. In the stationary phase, axenic growth was limited by peptone, glucose and yeast extract (DOM) (Fig. 4). Different *P. malhamensis* isolates have been shown to assimilate

the bulk of their required carbon via phagotrophy or osmotrophy and only to a minor extent by photo-autotrophy (Pringsheim 1952, Hutner et al. 1953, Caron et al. 1990, Sanders et al. 1990, Hahn & Höfle 1998, Zhang & Watanabe 2001, Boenigk et al. 2006). Our experiments support these results; however, DOM may contain additional nutrients that support osmotrophic growth; therefore, we tested glucose and glycerol as defined organic carbon sources. Osmotrophic dissimilation of glucose by *P. malhamensis* has been demonstrated within 1 to 2 d after transfer to an inorganic medium (Reazin 1956). In our experiments, only poor growth was observed with glucose ($\mu = 0.12 \pm 0.05$ d^{-1}) or glycerol ($\mu = 0.09 \pm 0.02$ d^{-1}) as a carbon source under axenic conditions (Fig. S7 in the Supplement) compared to growth with DOM ($\mu = 0.78 \pm 0.03$ d^{-1}) (Fig. 1, without agitation). However, the additional supply of glucose under nutrient limitation increased cell densities of *P. malhamensis* substantially and quickly (Fig. 4). This indicates the presence of additional growth-limiting substances, which might be present either in the yeast extract or the peptone of the DOM and which might be necessary for sustained osmotrophic growth of *P.*

malhamensis with glucose. For instance, citric acid cycle intermediates have been proposed to act in this way for *P. malhamensis* (Hutner et al. 1953). We can exclude soil extract in the standard BM medium as a source for growth-limiting substances as its omission did not affect growth rates or maximum cell densities of *P. malhamensis* during axenic cultivation.

The addition of peptone, glucose and yeast extract to cultures of *Mallomonas annulata* and *Dinobryon divergens* resulted in intense growth of the bacteria, making the determination of algal growth rates difficult. Thus, for these algae, we only tested particulate organic carbon sources. A summary for all 5 protists concerning the growth with different carbon sources is given in Table 2.

Influence of light

The role of light for growth of the protists was investigated by incubating the cells at different light:dark cycles and by measuring oxygen evolution using a Clark electrode (Table 2). Although oxygen evolution is often normalized to the chlorophyll content of the cells, we chose biovolume (μm^3) for normalization because a non-photosynthetic organism was included here. Maximum oxygen evolution rates of *Phaeodactylum tricornutum* (1.4 fmol O_2 μm^{-3} h^{-1}

or 80 fmol O_2 cell^{-1} h^{-1}; at pH 7 to 8, 20°C, 200 µmol photons m^{-2} s^{-1}) were similar to those obtained in other studies (~40 fmol O_2 cell^{-1} h^{-1} [Patel & Merrett 1986], ~20 to 100 fmol O_2 cell^{-1} h^{-1} [Müller & Wilhelm 1997]). All the photosynthetic algae in our study reached maximum oxygen evolution rates at light intensities between 150 and 200 µmol photons m^{-2} s^{-1} (Fig. 5).

As expected, *Mallomonas annulata* showed photo-autotrophic performance with maximum oxygen evolution rates of 1.48 ± 0.31 fmol O_2 μm^3 h^{-1}, which
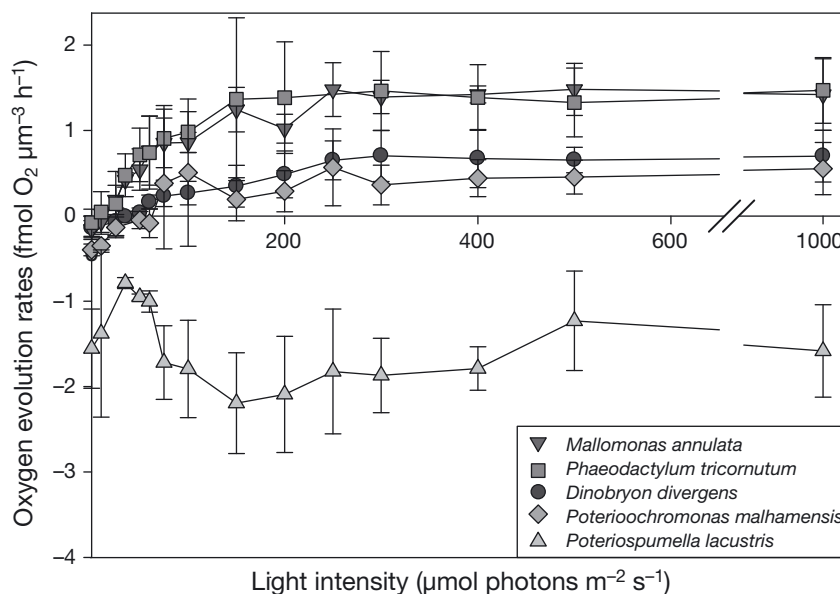

Fig. 5. Light-dependent oxygen evolution rates in late exponential growth phase (above zero: O_2 production, below zero: O_2 consumption) for the investigated chrysophytes and *Phaeodactylum tricornutum* standardized per biovolume and measured at different light intensities. Each data point represents the mean of 3 samples; error bars represent standard deviations

Table 2. Summary of the growth experiments. DOM: dissolved organic matter

Chrysophyte species	Growth in inorganic medium	Effect of aeration	Effect of DOM	Effect of bacteria	Effect of shortened light periods	Light-dependent oxygen evolution	Survival capacity in the dark
Phaeodactylum tricornutum	Yes	Increased maximum cell densities	Increased maximum cell densities	No effect	Decreased growth rates	Yes	Medium
Mallomonas annulata	Yes	Increased maximum cell densities	Not detectable	Decreased growth rates	Decreased growth rates	Yes	Low
Dinobryon divergens	Yes	Increased growth rates	Not detectable	Increased growth rates[a]	Decreased growth rates	Yes	Low
Poterioochromonas malhamensis	No	No effect	Increased growth rates	Increased growth rates	Reduced survival capacity	Yes	High
Poteriospumella lacustris	No	No effect	Increased growth rates	Increased growth rates	No effect	No	High

[a]Only with living bacteria added during the feeding experiment

is as high as the maximum oxygen evolution rates of *Phaeodactylum tricornutum* (1.46 ± 0.46 fmol O_2 μm^{-3} h^{-1}) (Fig. 5). Shortening of the photoperiod resulted in lower growth rates of both algae (Fig. 6). When cultures were shifted to shorter light periods, cell densities of *M. annulata* (6 h light) decreased by 32.3%, while *P. tricornutum* still showed positive growth rates at 4 h light. Thus, *P. tricornutum* is apparently better at coping with light limitation than *M. annulata* (see also Kamp et al. 2011, Rottberger et al. 2013).

While light is obviously indispensable for *Mallomonas annulata*, heterotrophic growth rates as well as maximum cell densities of *Poteriospumella lacustris* were similar in the light (μ = 0.63 ± 0.04) and in the dark (μ = 0.64 ± 0.04) (Fig. 6). Also, shortened photoperiods had no negative effects on growth in the presence of DOM. Oxygen evolution rates always remained negative due to respiration (−1.5 fmol O_2 μm^{-3} h^{-1}), independent of light (Fig. 5).

The mixotrophic *Poterioochromonas malhamensis* is able to grow heterotrophically in the dark and in the light. Holen (1999) showed that, different from other culture strains, the ingestion rates of bacteria by a freshly isolated *P. malhamensis* strain were affected by the light intensities applied. We did not observe differences between the growth rates of *P. malhamensis* in complete darkness and under the low-light conditions applied (Fig. 6). However, we found that the rate of photosynthetic oxygen evolution in *P. malhamensis* depends on light intensity (Fig. 5). Possibly the applied light intensity of 30 to 45 µmol quantum m^{-2} s^{-1} was too low to affect growth rates, and experiments under high-light conditions would have revealed a relationship between light intensity and growth rates.

Prolonged cell maintenance in the stationary phase is only possible if the metabolism can switch to other metabolic pathways that are not dependent on a limited resource or if enough storage
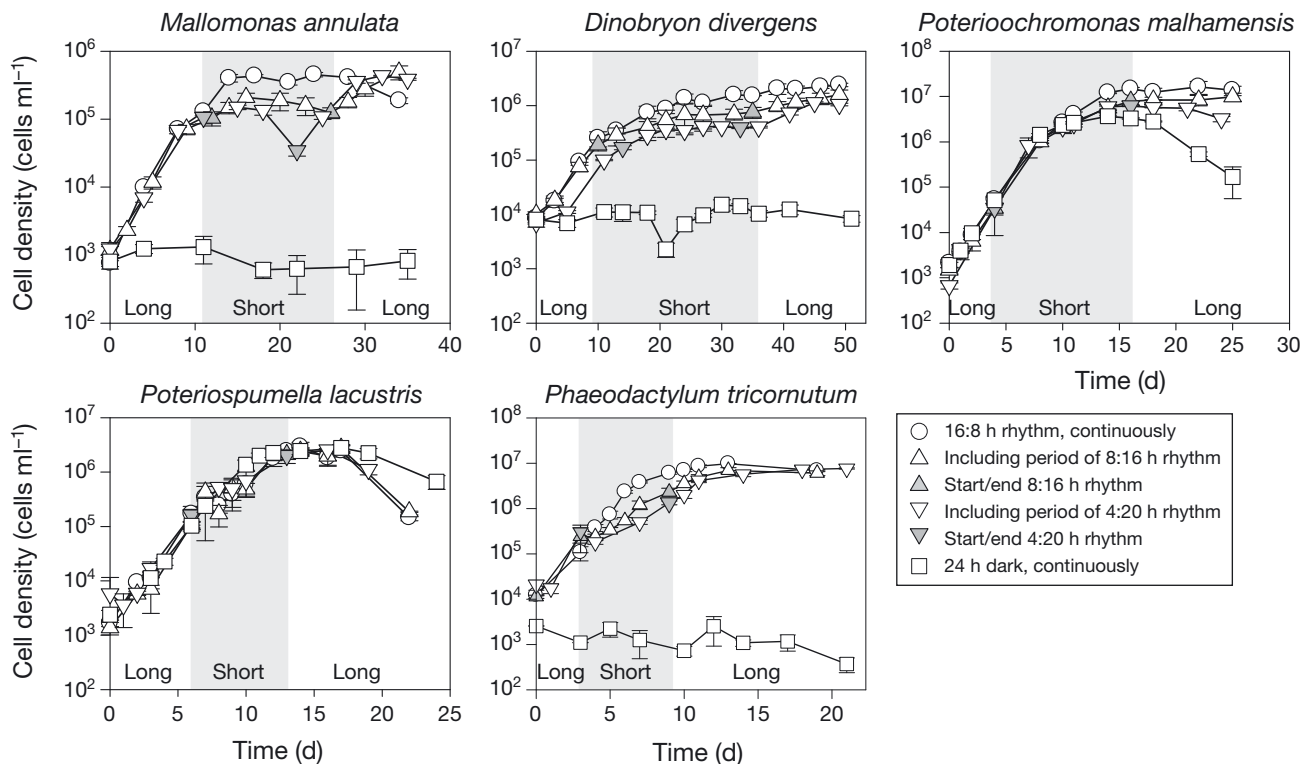

Fig 6. Growth of the investigated chrysophytes and *Phaeodactylum tricornutum* under different light regimes. Growth performance of cultures grown in a continuous 16 h light:8 h dark rhythm was compared to those partially grown in a 8 h light:16 h dark rhythm, a 4 h light:20 h dark rhythm and in continuous darkness. *Mallomonas annulata* was grown under a 6 h light:18 h dark rhythm instead of 4 h light:20 h dark. All cultures, besides those kept continuously in the dark, were initially incubated under 16 h light:8 h dark conditions (long). Light intervals were then shortened for the cells that were incubated with periods of 4 h (6 h) and 8 h light (short, grey shading), and afterward, the initial rhythm of 16 h light:8 h dark was re-applied (long). The grey shaded zone represents approximately the incubation period with shortened light intervals; the exact day of beginning and end of the incubation is indicated by filled symbols for each treatment. Each data point represents the mean of 3 samples; error bars represent standard deviations

resources for survival have been accumulated in the organism before. The period of stationary phase was rather short in dark experiments with *Poteriospumella lacustris* and *Poterioochromonas malhamensis* growing with DOM. Regarding *P. lacustris*, this is in agreement with the finding that *Spumella*-like flagellates are typical r-strategists, i.e. they have high growth rates when food is available but little starvation tolerance if food is absent (Boenigk et al. 2006). Cells of *P. malhamensis* grown with DOM survived much longer in the light than under dark conditions, while there was no difference for *P. lacustris* (Fig. 6). In contrast to *P. lacustris*, *P. malhamensis* is able to compensate organic carbon limitation in the light by switching to a phototrophic maintenance metabolism, which allows the alga to survive but not to proliferate in the presence of light. In the dark, this mechanism does not work, and the onset of cell mortality appears much earlier in the stationary phase (see also Caron et al. 1990, Zhang & Watanabe 2001). Altogether, our results show that *P. lacustris* is incapable of photoautotrophy, whereas *P. malhamensis* does not grow but sustains cell metabolism with the help of light-derived energy when organic carbon sources are limited.

Shortening of the light period resulted in lower growth rates of *Dinobryon divergens* (Fig. 6), and growth in the dark in the presence of particulate organic carbon was not observed (Fig. 2, Fig. S3). Oxygen evolution rates per biovolume were comparable to *Poterioochromonas malhamensis* (Fig. 5). Laboratory and field studies support our finding that *Dinobryon* is obligatorily light-dependent (Veen 1991, Caron et al. 1993, Watson et al. 2001, Watson & McCauley 2010).

Although *Mallomonas annulata* has higher maximum oxygen evolution rates than *Dinobryon divergens*, both algae have rather low growth rates compared to *Phaeodactylum tricornutum*. For unicellular organisms with photoautotrophic abilities, growth in terms of increasing cell densities or increasing cell size is coupled to the acquisition of inorganic carbon and oxygen production. There is, however, no direct relation between oxygen production, CO_2 consumption and growth. Oxygen is formed as a product of the photosynthetic light reaction, which provides energy and reduction equivalents for the complete formation of biomass by CO_2 assimilation. In addition to the direct formation of photosynthesis products, there are various direct or indirect metabolic sinks for the reduction equivalents generated in the photosynthetic light reaction. In addition to the direct formation of photosynthesis products, there are various direct or indirect metabolic sinks for the reduction equivalents generated in the photosynthetic light reaction. For example, the production of more reduced cell compounds (e.g. accumulation of lipids or synthesis of complex secondary metabolites), the secretion of carbohydrates or the nitrogen assimilation starting from nitrate can confound the stoichiometry between oxygen production and increase of biomass. Hence, the differences in growth rates observed for organisms with similar photosynthetic capacities are not necessarily a consequence of different carbon-uptake abilities or strategies. Mixotrophy furthermore increases the independence between growth and oxygen production. Thus, the non-linear relations comparing oxygen evolution rates and growth rates of *Poterioochromonas malhamenis*, *D. divergens*, *M. annulata* and *P. tricornutum* to each other might be a result of different exploitation of organic carbon sources but might also be related to differences in the budgeting of reduction equivalents.

SUMMARY

The present study provides profound insights regarding the nutrition of diverse chrysophytes by comparing them under identical conditions. Covering the whole spectrum from photoautotrophic to chemoheterotrophic chrysophytes, we found large differences between the more chemoheterotrophically mixotrophic chrysophyte *Poterioochromonas malhamensis* and the more photoautotrophically mixotrophic strain *Dinobryon divergens*. We demonstrated that both mixotrophic algae act within a certain nutritional spectrum, so that a total shift from purely heterotrophic to purely autotrophic growth or vice versa cannot be induced by varying light availability or different carbon sources. We also showed that bacteria promote growth of *P. malhamensis* and *D. divergens*, but that *P. malhamensis* proliferates independently from light availability, whereas *D. divergens* is obligatory light-dependent. *Mallomonas annulata* shows greater oxygen evolution rates under low-light conditions and, thus, different photosynthetic capacities than *D. divergens*. Individual growth responses regarding carbon and light availability were observed and, thus, a differential impact on the fitness of single chrysophytes in nature is anticipated.

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