Factors determining growth and vertical distribution of planktonic algae in extremely acidic mining lakes (pH 2.7)

Dissertation

Zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften

(Dr. rer. nat.)

in der Wissenschaftsdisziplin Gewässerökologie

am Institut für Biochemie und Biologie

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Potsdam

von Dipl. Biol. Vera Bissinger

geboren am 09.02.1968 in Rahden

Potsdam, im Februar 2003

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Tag der mündlichen Prüfung:	22. April 2003

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ABBREVIATIONS

AP	alkaline phosphatase
APA	alkaline phosphatase activity
C	carbon
Chl	chlorophyll *a*
DCM	deep chlorophyll maximum
DOC	dissolved organic carbon
DOP	dissolved organic phosphorus
ELF	enzyme labeled fluorescence
IC	inorganic carbon
μ/I	growth/Irradiance
M	molar (mol L^{-1})
ML	mining lake
P	phosphorus
PAR	photosynthetic available radiation
P/I	Production (*here :* O_2)/Irradiance
SRP	soluble reactive phosphorus
TBS	Tagebausee
TP	total phosphorus

ZUSAMMENFASSUNG

Die vorliegende Dissertation beschäftigt sich mit den Faktoren, die das Wachstum und die Vertikalverteilung von Planktonalgen in extrem sauren Tagebaurestseen (TBS; pH 2-3) beeinflussen. Im exemplarisch untersuchten TBS 111 (pH 2.7; Lausitzer Revier) dominiert die Goldalge *Ochromonas* sp. in oberen und die Grünalge *Chlamydomonas* sp. in tieferen Wasserschichten, wobei letztere ein ausgeprägtes Tiefenchlorophyll-Maximum (DCM) ausbildet. Es wurde ein deutlicher Einfluss von Limitation durch anorganischen Kohlenstoff (IC) auf das phototrophe Wachstum von *Chlamydomonas* sp. in oberen Wasserschichten nachgewiesen, die mit zunehmender Tiefe von Lichtlimitation abgelöst wird. Im Vergleich mit Arbeiten aus neutralen Seen zeigte *Chlamydomonas* sp. erniedrigte maximale Wachstumsraten, einen gesteigerten Kompensationspunkt und erhöhte Dunkelrespirationsraten, was auf gesteigerte metabolische Kosten unter den extremen physikalisch-chemischen Bedingungen hinweist. Die Photosyntheseleistungen von *Chlamydomonas* sp. waren in Starklicht-adaptierten Zellen durch IC-Limitation deutlich verringert. Außerdem ergaben die ermittelten minimalen Zellquoten für Phosphor (P) einen erhöhten P-Bedarf unter IC-Limitation. Anschließend konnte gezeigt werden, dass *Chlamydomonas* sp. ein mixotropher Organismus ist, der seine Wachstumsraten über die osmotrophe Aufnahme gelösten organischen Kohlenstoffs (DOC) erhöhen kann. Dadurch ist dieser Organismus fähig, in tieferen, Licht-limitierten Wasserschichten zu überleben, die einen höheren DOC-Gehalt aufweisen. Da die Vertikalverteilung der Algen im TBS 111 jedoch weder durch IC-Limitation, P-Verfügbarkeit noch die in situ DOC-Konzentrationen abschließend erklärt werden konnte (*bottom-up* Kontrolle), wurde eine neue Theorie zur Entstehung der Vertikalverteilung geprüft. *Grazing* der phagotrophen und phototrophen Alge

Ochromonas sp. auf der phototrophen Alge *Chlamydomonas* sp. erwies sich als herausragender Faktor, der über *top-down* Kontrolle die Abundanz der Beute in höheren Wasserschichten beeinflussen kann. Gemeinsam mit der Tatsache, dass *Chlamydomonas* sp. DOC zur Wachstumssteigerung verwendet, führt dies zu einer Akkumulation von *Chlamydomonas* sp. in der Tiefe, ausgeprägt als DCM. Daher erscheint *grazing* als der Hauptfaktor, der die beobachtete Vertikalschichtung der Algen im TBS 111 hervorruft. Die erzielten Ergebnisse liefern grundlegende Informationen, um die Auswirkungen von Strategien zur Neutralisierung der TBS auf das Nahrungsnetz abschätzen zu können.

SUMMARY

In this thesis, I investigated the factors influencing the growth and vertical distribution of planktonic algae in extremely acidic mining lakes (pH 2-3). In the focal study site, Lake 111 (pH 2.7; Lusatia, Germany), the chrysophyte, *Ochromonas* sp., dominates in the upper water strata and the chlorophyte, *Chlamydomonas* sp., in the deeper strata, forming a pronounced deep chlorophyll maximum (DCM). Inorganic carbon (IC) limitation influenced the phototrophic growth of *Chlamydomonas* sp. in the upper water strata. Conversely, in deeper strata, light limited its phototrophic growth. When compared with published data for algae from neutral lakes, *Chlamydomonas* sp. from Lake 111 exhibited a lower maximum growth rate, an enhanced compensation point and higher dark respiration rates, suggesting higher metabolic costs due to the extreme physico-chemical conditions. The photosynthetic performance of *Chlamydomonas* sp. decreased in high-light-adapted cells when IC limited. In addition, the minimal phosphorus (P) cell quota was suggestive of a higher P requirement under IC limitation. Subsequently, it was shown that *Chlamydomonas* sp. was a mixotroph, able to enhance its growth rate by taking up dissolved organic carbon (DOC) via osmotrophy. Therefore, it could survive in deeper water strata where DOC concentrations were higher and light limited. However, neither IC limitation, P availability nor in situ DOC concentrations (bottom-up control) could fully explain the vertical distribution of *Chlamydomonas* sp. in Lake 111. Conversely, when a novel approach was adopted, the grazing influence of the phagotrophic phototroph, *Ochromonas* sp., was found to exert top-down control on its prey (*Chlamydomonas* sp.) reducing prey abundance in the upper water strata. This, coupled with the fact that *Chlamydomonas* sp. uses DOC for growth, leads to a pronounced accumulation of *Chlamydomonas* sp. cells at depth; an apparent DCM. Therefore, grazing appears to be the

main factor influencing the vertical distribution of algae observed in Lake 111. The knowledge gained from this thesis provides information essential for predicting the effect of strategies to neutralize the acidic mining lakes on the food-web.

1 GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Introduction

In the former lignite mining regions of eastern Germany, hundreds of lakes have been created, and are still forming, in abandoned open-cast mines due to ground- and rain water inputs (Friese et al. 1998, Schultze et al. 1999). The weathering and oxidation of the sulfide minerals (pyrite, marcasite) associated with lignite when exposed to air and water, have geochemically acidified these lakes (Stumm and Morgan 1970). The lakes are now characterized by their extremely low pH (2.0-3.5) and represent some of the most acidic waters in the world (Geller et al. 1998). They also have extremely high total iron (up to 300 mg l^{-1}), aluminum (up to 40 mg l^{-1}) and sulfate (up to 4000 mg l^{-1}) concentrations, and, unlike circum-neutral lakes in which HCO_3 is the dominant ion (Geller et al. 1998), inorganic carbon (IC) is available in low concentrations only as CO_2 (< 0.5 mg l^{-1} in the epilimnion). The high iron content typically causes the red coloration of the water (Photograph 1-1) and modifies the vertical light spectrum along the water column (Koschorreck and Tittel 2002). Picture 1-1 depicts the focal study site for this investigation, the acidic mining Lake 111 in the Lusatian District (Lake 111; pH 2.7).

As a consequence of the extreme physico-chemical conditions, Lake 111 supports only a low diversity, microbial community of bacteria, algae and Protozoa (ciliates and Heliozoa) plus low numbers of Metazoa (two species of rotifers) (Wollmann et al. 2000). The biomass in the pelagic is entirely dominated by two pigmented, flagellated algae, the chlorophyte *Chlamydomonas* sp. and the chrysophyte *Ochromonas* sp. Together these represent over 90% of the total biomass.

Picture 1-1: The red coloration of acidic mining lake 111 (Lake 111). Photograph by E. Bell.

The algae exhibit a distinct vertical distribution in Lake 111. *Ochromonas* sp. dominates in the upper water strata and *Chlamydomonas* sp. dominates at depth, forming a pronounced deep chlorophyll maximum (DCM) (Kapfer et al. 1997, Nixdorf et al. 1998). The mechanisms giving rise to the vertical distribution of these algae are far from being understood. Despite its key role in the pelagic food web of the mining lakes, detailed studies on the growth and photosynthetic rates of *Chlamydomonas* sp. under in situ conditions were entirely lacking. Thus, the main aim of this study was to investigate the factors that influence the algal distribution observed in Lake 111 and relate the findings to the lake's physico-chemical conditions.

Until now, the low IC concentrations together with phosphorus (P) availability in the epilimnion have been used to explain the vertical distribution of algae observed in Lake 111 (Nixdorf et al 1998, Lessmann et al. 1999, Beulker et al. 2002). However, in situ, the vertical gradient of IC in Lake 111 does not correspond with the realized *Chlamydomonas* cell densities and inorganic nutrient limitation, and therefore cannot be used to explain the high

cell densities observed in deeper strata. Furthermore, results from previous investigations on potential P limitation in acidic mining lakes are contradictory. P enrichment experiments in Lake 111 resulted in enhanced primary production rates (Beulker et al. 2002), but the un-enriched, in situ soluble reactive phosphorus (SRP) concentration throughout the water column is already above the threshold value commonly assumed to limit phytoplankton growth in most algal species and should therefore be sufficient to support a higher algal standing stock in the epilimnion than is actually realized (Sas 1989). This study therefore aimed first to experimentally clarify what influence IC and P have on the growth and photosynthesis of *Chlamydomonas* sp. (Chapters 3, 4 and 5), before proceeding to identify other factors.

Many species from the genera *Ochromonas* and *Chlamydomonas* exhibit a mixotrophic potential (Rothhaupt 1996a, Laliberté and Noüe 1993), thus, more than 90% of the pigmented organisms in Lake 111 are potentially mixotrophs. Mixotrophs are known to be able to cover their carbon and energy demands via both photosynthesis and the uptake of organic carbon in a particulate or dissolved form. For example, many chrysomonads, like *Poterioochromonas malhamensis* or other *Ochromonas* species from circumneutral habitats, are known to take up bacteria and algae, supplementing photosynthesis with phagotrophic nutrition (Aaronson 1974, Keller et al. 1994, Rothhaupt 1996a, Sanders et al. 2001, Zhang and Watanabe 2001). Conversely, Chlamydomonas species in a number of aquatic environments are known to use dissolved organic carbon sources (osmotrophy) (Bennett and Hobbie 1972, Laliberté and Noüe 1993). Referring to the mixotrophic gradient of Jones (2000), *Ochromonas* spp. lies toward the heterotrophic, and *Chlamydomonas* spp. toward the phototrophic end of the mixotrophic gradient. Mixotrophy has not yet been demonstrated in either alga in Lake 111 or any other acidic mining lake but could influence their vertical distribution. Circumstantial

evidence suggests that this could be the case. For example, DOC levels increase with increasing depth in Lake 111; therefore, if *Chlamydomonas* sp. were able to supplement photosynthesis with the uptake of DOC, this would explain its dominance in deeper strata. Chapter 3 investigates the mixotrophic potential of *Chlamydomonas* sp. and relates this to the vertical distribution of the alga.

The final factor investigated as potentially influencing the vertical distribution of *Chlamydomonas* sp. observed in Lake 111 is top-down control by predators/ grazers. The formation of DCM has traditionally been interpreted as a consequence of nutrient availability in deeper strata (bottom-up control) (Reynolds 1997). However, in this study, a novel approach has been adopted to demonstrate that the DCM may not simply reflect an accumulation of algal biomass at depth but rather the removal of algal biomass in upper water strata by grazers. This mechanism was experimentally tested and the results presented in Chapter 6 challenge existing ecological paradigms concerning the formation of DCM in aquatic environments.

In order to achieve the aims discussed above and investigate the factors influencing the growth and photosynthesis of mining lake algae, a significant part of the study was spent modifying existing techniques and developing novel methods to simulate the extreme physico-chemical conditions of Lake 111 in the laboratory. This included i) the development of a culture medium that reflected the chemical conditions in Lake 111. The medium had to be free of organic carbon sources to enable investigations of purely autotrophic, and defined mixotrophic and heterotrophic growth of phytoplankton organisms. ii) The isolation and culturing of the dominant plankton organisms present in Lake 111. The *Chlamydomonas* and *Ochromonas* strains were subsequently provided to the SAG (Sammlung für Algenkulturen,

Göttingen) for public use. iii) The development of a special light-box which simulated the shifted light spectrum in the mining lakes whilst maintaining a controlled, stable temperature. In addition, a device was included to regulate inorganic carbon concentration in the cultures. These are described in detail in Chapters 2 and 3.

Outline of thesis

The overall aim of this thesis was to characterize the factors determining growth and vertical distribution of planktonic algae in extremely acidic mining lakes (pH 2.7), in particular *Chlamydomonas* sp., from mining Lake 111. In the following chapter (Chapter 2) I describe the abovementioned methodological developments. Chapter 3 is the most extensive chapter in this thesis. It addresses the questions: Is autotrophic growth in mining lakes limited by the low inorganic carbon concentrations in the epilimion? Is *Chlamydomonas* sp. able to use DOC for growth (mixotrophy)? Does the use of DOC lead to higher growth rates, especially under IC limitation? Does the catabolism of exogenous organic carbon provide intracellular CO_2 which stimulates photosynthesis under IC depletion? And, do the adverse environmental conditions increase metabolic costs and reduce maximum growth? Chapter 4 further investigates the questions in Chapter 3 through the investigation of the photosynthetic potential of *Chlamydomonas* sp. under different light intensities and supplies of IC and DOC. In Chapter 5, the application of new methods for the detection of P depletion in single cells under the adverse conditions was tested. The final experimental chapter, Chapter 6, presents a novel theory to explain the vertical distribution of the algae in Lake 111, especially the formation of the DCM, via grazing (top-down control), rather than resource availability (bottom-up control). The final Chapter 7 summarizes and discusses the results of this thesis, places them in a regional context and discusses their ecological implications.

2 ESTABLISHING THE PREREQUISITES FOR GROWTH EXPERIMENTS IN THE SPECIFIC ENVIRONMENT

A new medium free of organic carbon to cultivate organisms from extremely acidic lakes (pH 2.7)

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published in

Acta hydrochim. hydrobiol. 28:310-312, 2000.

ABSTRACT

An algal culture medium was developed which reflects the extreme chemical conditions of acidic mining lakes (pH 2.7, high concentrations of iron and sulfate) and remains stable without addition of organic carbon sources. It enables controlled experiments e.g. on the heterotrophic potential of pigmented flagellates in the laboratory. Various plankton organisms isolated from acidic lakes were successfully cultivated in this medium. The growth rates of a *Chlamydomonas*-isolate from acidic mining lakes were assessed by measuring cell densities under pure autotrophic and heterotrophic conditions (with glucose as organic C-source) and showed values of 0.74 and 0.40, respectively.

INTRODUCTION

Highly acidic environments are numerous on earth and gain increasingly importance owing to the creation of new lake districts by the reduction of open cast lignite mining activities e. g. in Eastern Germany. These geogenically acidified lakes have an extremely low pH (2.5…3.5) and high iron (up to 500 mg L^{-1}) and sulfate concentrations (up to 4000 mg L^{-1}) (Klapper and Schultze, 1995). The understanding of their plankton food web dynamics is a challenge for basic research on extreme environments and urgently needed for proper management.

The phytoplankton in acidic environments is frequently dominated by potentially mixotrophic species (*Chlamydomonas*, *Ochromonas*) which has been considered as a successful strategy to face the frequently encountered low inorganic carbon concentrations (Nixdorf and Kapfer, 1997). Knowledge on the contribution of organic carbon to the nutrition of the pigmented flagellates cannot be inferred from literature data as it is species-specific and highly dependent on environmental conditions. It is, however, required for the fundamental understanding of ecosystem functioning which forms the basis to improve technologies for neutralization of the acidic waters by addition of organic carbon.

In order to study the heterotrophic potential of the pigmented flagellates under defined environmental conditions, a culture medium is required which is free from organic carbon sources and reflects in situ conditions. Olaveson and Stokes (1989) designed a modified acid medium (MAM) simulating acid mine drainage waters with a pH of 4.0 to investigate the effects of inorganic and organic carbon additions on the growth rate of *Euglena mutabilis*. The extremely acidic mining lakes are buffered by the $Fe(OH)_x$ system (Schultze and Geller, 1996) and small changes in the pH can cause precipitation of $Fe(OH)_3$. Hence, Olaveson and Stokes added organic substances such as EDTA to their medium in order to prevent the precipitation of iron and to keep it autoclavable. However, these organic substances may complicate the interpretation of experiments concerning the heterotrophic growth of

mixotrophic phytoplankton. Gimmler and Weis (1992) composed a medium suitable for *Dunaliella acidophila* from sulfuric soils and springs (pH 0.3 – 3.0) without organic carbon but extremely low iron concentrations (0.02 mM). The medium presented here is free of organic carbon and contains 2.6 mM iron and, thus, reflects better the extreme chemical situation in the mining lakes and enables the investigation of the heterotrophic potential of algae with defined additions of organic carbon.

PREPARATION OF THE MEDIUM

We selected ion concentrations in our medium (medium 111, Table 2-1) similar to those found in a representative mining lake (ML111 in the Lusatian region, Germany). In contrast to in situ conditions phosphorus concentrations were enhanced and aluminum concentrations reduced in order to exclude phosphorus limitation due to chemical complexation of phosphorus by aluminum (Joseph et al. 1995).

Table 2-1. Comparison of the main ion concentrations (mmol L^{-1}) in the epilimnion of mining lake 111 (Wiegleb 1998) and in the medium 111.

	Mining lake 111	**Medium 111**
pH	2.6±0.08	2.65
Ca^{2+}	5.65	7.47
Mg^{2+}	1.19	1.16
Na^{+}	<0.4	0.26
K^{+}	0.10	0.07
$Fe_{ges.}$	2.79	2.60
Al^{3+}	1.41	0.74
Mn^{2+}	0.06	0.05
$(NH_4)^{+}$	0.20	0.16
Cl^{-}	0.24	0.26
$(SO_4)^{2-}$	13.65	13.74
$(NO_3)^{-}$	0.02	0.02
TP	0.0004	0.05

The following steps are required to prepare the medium (Table 2-2):

Stock solutions:

1. Separate stock solutions for each of the substances No. 3…9.
2. Combined stock solution of the trace elements (No. 10…21)

Preparation of the medium (adhere strictly to the given order):

3. Stir 1287 mg $CaSO_4 \cdot 2H_2O$ in ca. 800 mL aqua bidest. until it is completely dissolved.
4. Add 1 mL 1 N H_2SO_4.
5. Put in 519 mg $Fe_2(SO_4)_3$ and stir until it is completely dissolved.
6. Add the amount of stock solutions of the substances 3…8 as given in Table 2-2 and 0.4 mL of the trace elements stock solution.
7. Adjust the pH to 2.65 with H_2SO_4 (1 N).
8. Add stock solution No. 9 dropwise while stirring thoroughly.
9. *modification with glucose:*

   Addition of Alpha-D-Glucose up to a concentration of 20 mg C L^{-1}.
10. Add aqua bidest. to 1000 mL.
11. Immediately sterilize the medium by filtration through cellulose-acetate-filter with a pore size of 0.2 µm (SARTORIUS, order no.11107-50-N), reject the first 100 mL of the filtrate. Do not autoclave!

We observed no precipitation of $Fe(OH)_3$ at a pH between 2.6…2.7.

Table 2-2. Composition of medium 111, pH 2.65, designed for growth experiments with plankton from acidic mining lakes.

No.	*Substance* (p.a.-quality)	*Mmol per liter medium*	*Concentration in the stock solution (mg per 250 mL stock)*	*mL stock per liter medium*
1	$CaSO_4 \cdot 2H_2O$	7.474	-	-
2	$Fe_2(SO_4)_3$	1.299	-	-
3	NaCl	0.260	759.72	5
4	$MgSO_4 \cdot 7H_2O$	1.155	7117.11	10
5	KNO_3	0.020	505.55	1
6	K_2SO_4	0.00023	106.24	0.1
7	$(NH_4)_2SO_4$	0.082	270.89	10
8	$Al_2(SO_4)_3 \cdot 16H_2O$	0.371	29224.85	2
9	KH_2PO_4	0.050	3402.25	0.5
	Stock solution of trace elements			0.4

Stock solution of trace elements (modified after Zehnder and Gorham, 1960):

No.	*Substance* (p.a.-quality)	*µmol per liter medium*	*Concentration stock solution (mg per 500 mL stock)*
10	$Na_2WO_4 \cdot 2H_2O$	0.00788	3.25
11	$Na_2MoO_4 \cdot 2H_2O$	0.04001	12.10
12	KBr	0.07991	11.88
13	KI	0.03982	8.25
14	$Co(NO_3)_3 \cdot 6H_2O$	0.04020	14.63
15	$CuSO_4 \cdot 6H_2O$	0.04005	12.50
16	$NiSO_4 \cdot 7H_2O$	0.04020	14.10
17	$VOSO_4 \cdot 2H_2O$	0.00603	1.50
18	H_2SeO_3	0.06017	9.70
19	H_3BO_3	4.011	310.00
20	$MnSO_4 \cdot H_2O$	50.8	5366.50
21	$ZnSO_4 \cdot 7H_2O$	13.761	4946.00

ASSESSMENT OF GROWTH RATES OF *CHLAMYDOMONAS* SP. WITH THE NEW MEDIUM

Various plankton organisms such as pigmented flagellates (*Chlamydomonas* sp., *Ochromonas* sp.), chlorococcal algae, ciliates (*Oxytricha* sp.) and rotifers (*Cephalodella* sp.) were isolated from acidic mining lakes and successfully cultivated in this medium.

The auto- and heterotrophic potential of *Chlamydomonas* sp. was investigated in semicontinuous cultures (40% replacement per day) with and without addition of glucose in the dark. Once a day the density of the algae was controlled photometrically (750 nm) and converted to cell numbers with standard curves. First, six culture vessels were incubated at 20°C in the light without glucose for 11 days until the cells grew stable. From day 12 onwards all vessels were incubated in the dark, three with and three without an addition of α-D-glucose (20 mg C L^{-1}).

All experiments were conducted under controlled conditions (20° ± 2° C, light cultures obtained 70 µE m^{-2} s^{-1} provided by cool white neon tubes and a 16:8 h LD photoperiod). The cultures were rotated every three hours.

Growth rates were derived by fitting linear regression lines to the logarithm of the cell concentrations observed at day 2…10 (light), day 15…22 (dark with glucose), and day 13…18 (dark without glucose) (Fig. 2-1) (after Fencl et al. 1961).

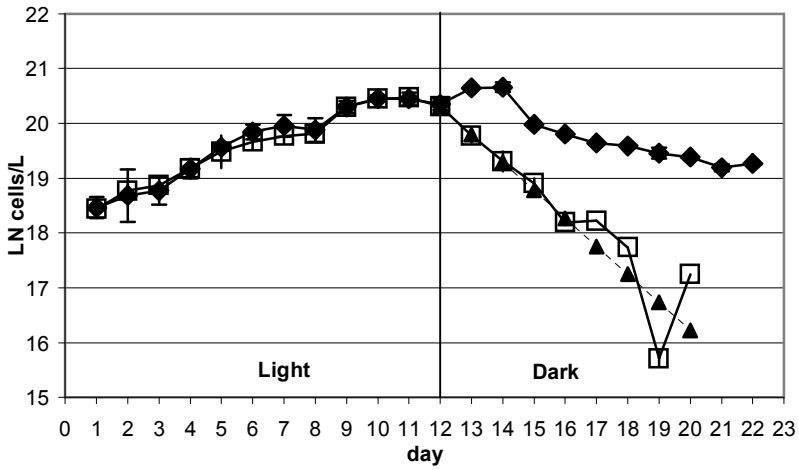

Fig. 2-1. Development of *Chlamydomonas* cell cultures in medium 111, pH 2.65, under semicontinuous conditions (40% replacement per day) in the light (day 1…12, without glucose) and in the dark (day 13…22) with glucose addition (filled rhombic symbols) and without glucose addition (light squares). The theoretical cell decrease according to the dilution rate is depicted by triangles. Mean values of 3 parallels, bars indicate standard deviations (not visible bars are smaller than the symbols).

RESULTS AND DISCUSSION

In the light, *Chlamydomonas* sp. had a specific growth rate of 0.74 per day (doubling time: 22.5 h, day 2…10) (Fig. 2-1). In the dark with addition of glucose growth rates of 0.40 per day (doubling time: 1.7 days) (day 15…22) were measured. In the dark without glucose no growth was observed as cell numbers decreased according to the dilution rate, until their numbers were too low to allow precise measurements (from day 19 onwards).

Our *Chlamydomonas*-strain grew faster in the light than heterotrophically in the dark, but showed modest growth rates with high DOC-concentrations. Our results suggest that the *Chlamydomonas*-strain, isolated from ML111, has a heterotrophic potential under the given acidic conditions.

CONCLUSIONS

The experimental results proved the applicability of the newly designed medium for conducting growth experiments with organisms from extremely acidic aquatic environments, such as mining lakes, to study their physiological and ecological properties under defined laboratory conditions.

ACKNOWLEDGEMENTS

We thank P. Herzsprung and R. Kringel for their chemical advices and U. Gaedke and G. Weithoff for revising the manuscript.

3 GROWTH EXPERIMENTS

Mixotrophic growth with natural dissolved organic carbon in *Chlamydomonas* sp. from an acidic lake (pH 2.6) under CO_2 and light limitation

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Submitted to

Limnology and Oceanography

ABSTRACT

Plankton communities in acidic mining lakes are species-poor because they face extreme environmental conditions (pH 2.5-3.3, e.g. 16 mmol L^{-1} SO_4^{2-}, 3 mmol L^{-1} Fe^{2+} + Fe^{3+}). We measured the growth rates of the dominant phytoplankton species, *Chlamydomonas* sp., in semi-continuous laboratory culture experiments and in situ enrichment experiments in two lakes. The following hypotheses were tested: (H-1) low inorganic carbon (IC) concentrations in the epilimnion (e.g. 25 µmol L^{-1}) arising from the low pH limit photosynthesis; (H-2) the additional use of dissolved organic carbon (mixotrophy) leads to higher growth rates under IC limitation; (H-3) the catabolism of exogenous organic carbon provides intracellular CO_2 which stimulates photosynthesis; and (H-4) the adverse environmental conditions increase metabolic costs and reduce maximum growth. (H-1) was tested in culture experiments. These indicated IC limitation of *Chlamydomonas* in the mixed epilimnion, but light limitation below.

(H-2) was confirmed as well; in the laboratory, mixotrophic growth always exceeded pure phototrophic growth. Furthermore, significant dark growth in filtered lake water provided unequivocal evidence that *Chlamydomonas* was able to use the natural DOC available in the lake. (H-3) was rejected since growth was enhanced by glucose even when photosynthesis was not limited by CO_2. (H-4) was accepted due to low maximum phototrophic and mixotrophic growth, and a high compensation light intensity compared to algae in non-acidic waters. *Chlamydomonas* exhibited starvation resistance in the dark (no reduction of cell number or size over 28 days, 20°C). *Chlamydomonas*, a typical *r*-strategist in circum-neutral systems, exhibited characteristics of a *K*-strategist in the stable, acidic lakes environment investigated.

INTRODUCTION

After the cessation of open cast lignite mining in Eastern Germany, more than 150 lakes, often of remarkable size, depth, and economical potential, formed and are still forming in the abandoned mine pits. Many of these lakes have been geogenically acidified due to weathering of pyrite and marcasite (FeS_2), resulting in a pH of between 2.5 and 3.3. The lakes contain high iron concentrations (e.g. 2.7 mmol L^{-1}) and are consequently highly pigmented (orange-red), modifying the underwater light spectrum (UV and blue light absorbed in the upper strata).

As a consequence of the low pH the inorganic carbon (IC) exists almost exclusively as dissolved CO_2 (Stumm and Morgan 1970) and a bicarbonate pool is lacking. CO_2 concentrations in upper strata of acidic lakes (25-41 µmol L^{-1} in the epilimnion) are near the theoretical equilibrium with the atmosphere (13 µmol L^{-1}, 20°C; Satake and Saijo 1974) and so low that they have the potential to limit photosynthetic growth (Ohle 1981). The CO_2 concentrations are in the range of the half-saturation concentration of Rubisco

(ribulosebisphosphate carboxylase/oxygenase), the "key"-enzyme of photosynthetic C-fixation, which lies for algae between 15 and 200 µmol L^{-1} (Moroney, 2001). In deep water layers IC concentrations up to 4000 µmol L^{-1} were measured, presumably due to respiration processes in the lake (Satake & Saijo 1974) or from groundwater input.

Generally, more than 90% of the biomass of pigmented organisms in the acidic lakes of this area consists of potential mixotrophs. These mixotrophs are known to be able to cover their carbon and energy demands via both photosynthesis and the uptake of organic carbon. The relative contribution of mixotrophs to phytoplankton far exceeds what would be expected in circum-neutral, temperate lakes. To a lesser extent than in the acidic lakes, a relatively high proportion of mixotrophic biomass is also found in other systems characterized by low under water light (deep water layers, turbid reservoirs, humic lakes, under snow and ice, at extremely high latitudes, or in dense algal populations) (Berninger et al. 1992; Wetzel 2001). In the acidic mining lakes little is known about mixotrophic resource utilization. The dominant pigmented species are represented by *Chlamydomonas* sp. (Chlorophyceae) and *Ochromonas* sp. (Chrysophyceae) (Nixdorf et al. 1998). According to the classification of Jones (2000), *Chlamydomonas* typically relies on phototrophic nutrition, supplementing its carbon/energy budget with osmotrophy (uptake of dissolved organic carbon, DOC). For example, *C. humicola* exhibited its highest growth rates in the light with acetate additions (Laliberté and Noüe 1993). Some *Chlamydomonas* species are known to be acid tolerant, e.g. *C. acidophila* from acidic volcano and mining lakes (Erlbaum Cassin 1974; Gyure et al. 1987). *Chlamydomonas* was chosen to investigate the following four hypotheses (H-1 – H-4), because it represents the dominant photosynthetic organism in our extremely acidic lakes:

(H-1) Low IC concentrations in the epilimnion limit photosynthesis.

Ohle (1981) observed a positive correlation between the quantity of algal carbon assimilation and the IC concentration in acidic lake water. Furthermore, indirect evidence for IC limitation

of the autotrophic phytoplankton in acidic lakes has been derived from increased phytoplankton biovolume under ice cover, attributed to accumulation of IC (Wollmann et al. 2000). Lessmann et al. (1999) observed a positive correlation between biomass distribution and IC concentration in the acidic mining lakes and subsequently proposed the existence of special mechanisms for carbon acquisition in the algal species without providing further evidence to prove the impact of IC depletion on phytoplankton growth. Olaveson and Stokes (1989), observed enhanced growth of *Euglena mutabilis* with CO_2-enrichment in the laboratory, but their medium contained organic buffers which interfere with osmotrophic nutrition. Due to the low IC concentrations in the investigated acidic lakes we hypothesized that photosynthetic growth would be limited in the epilimnion of our study lakes.

(H-2) Mixotrophic nutrition leads to higher growth rates under IC limitation.

The overwhelming contribution of mixotrophs to the eukaryotic plankton in acidic lakes raises the question of what gives them a competitive advantage over purely phototrophic and heterotrophic flagellates. The mixotrophs could gain an advantage by supplementing their nutritional requirements, e.g. by obtaining phosphorus via bacterivory, or by supplementing their carbon and energetic requirements by taking up, for example, DOC at low light intensities. However, mixotrophs must also maintain energy expensive photosynthetic apparatus, placing them at a competitive disadvantage in situations where resources promote high phototrophic or heterotrophic growth in specialists (Rothhaupt 1996a and b). Nevertheless, by combining resources, mixotrophs survive at low resource concentrations exhibiting low growth and low population loss rates. We hypothesize that *Chlamydomonas* supplements its autotrophic growth through DOC uptake and that the predominance of mixotrophs observed in the acidic lakes results from the fact that they are competitively superior under low IC conditions which limit photosynthetic growth.

(H-3) The catabolism of exogenous organic carbon provides intracellular CO_2 which stimulates photosynthesis.

In contrast to (H-2) which refers to a pure heterotrophic use of DOC in addition to phototrophic growth, here, we hypothesize the oxidation of DOC and stimulation of the Calvin-cycle by internally produced CO_2. Vincent and Goldman (1980) observed that exogenous ^{14}C-labelled organic substrates were incorporated in algal cells at a light dependent rate and hypothesized that these were catabolized to CO_2 which was subsequently photosynthetically refixed while still within the cell. Indeed, it is known that algae grown at low light intensities can respire exogenous organics almost completely (Lewitus and Kana 1994), and Villarejo et al. (1995) demonstrated that *Chlorella vulgaris* refixed catabolized CO_2 during photosynthesis when grown under neutral, IC limiting conditions with light saturation.

(H-4) The adverse environmental conditions in the acidic lakes increase metabolic costs and reduce maximum growth.

Phytoplankton can develop special adaptations under acidic conditions. At pH 1.0, *Dunaliella acidophila* maintains a positive membrane potential to avoid the inflow of H^+ ions into the cell and maintain an internal pH of 7.0. This mechanism may require special ATP-driven ion pumps in the membrane (Gimmler & Weis 1992). Furthermore, extracellular enzymes, like hydrolases or phosphatases, may play an important role for nutrient acquisition under the adverse conditions (Gross 2000). These additional costs for maintenance under the high proton and extreme ionic concentrations are likely to affect maximum growth rates and other characteristics of growth-light-relationships, such as the compensation light intensity. Indeed, Turner et al. (1991), measured higher dark respiration rates and higher compensation irradiances in benthic communities in an experimentally acidified lake as compared with a neutral system, suggesting higher metabolic costs in the acidic environment. Overall, we

hypothesized that growth-light-curves would reflect special algal characteristics under the extreme conditions, such as a reduced photosynthetic quantum efficiency, lowering maximum growth and increasing the minimum light intensity required to obtain zero cellular growth.

To test the four hypotheses we conducted both field experiments and laboratory experiments using *Chlamydomonas* isolated from an acidic lake. The nutrients N and P were assumed to be non-limiting in the investigated lakes since ammonia concentrations were high (Table 3-1) and soluble reactive phosphorus (SRP) concentrations consistently surpassed 0.2 µmol L^{-1}. In growth experiments conducted with filtered lake water and P-enriched medium we obtained similar growth rates, indicating sufficient mineral nutrient concentrations.

Table 3-1. Morphometric, physical and chemical parameters in the epi- or mixolimnion of the investigated lakes. Detailed physical and chemical description available in Herzsprung et al. 1998, Friese et al. 1998 and 2002.

	Lake 111	Lake Niemegk
Formation year	1958	1995
Area (km^2)	0.11	0.73
Volume (10^6 m^3)	0.5	4.0
Mean depth (m)	4.7	5.7
Maximum depth (m)	10	15
Secchi depth (m)	3.5	0.5-1.2
Conductivity (mS cm^{-1})	2.60	3.03
pH	2.66	2.85-2.94
SO_4^{2-} (mmol L^{-1})	13.85	17.44
Al^{3+} (mmol L^{-1})	1.47	0.63
Fe^{2+} (mmol L^{-1})	0.04	0.02
Fe^{2+} + Fe^{3+} (mmol L^{-1})	2.83	1.93

In situ, we investigated the effect of additions of IC, DOC, dissolved organic phosphorus (DOP), and of dark incubation on phytoplankton growth to improve our understanding of growth determining factors. In the laboratory, growth rates were determined as close to in situ conditions as possible, with special emphasis on the impact of light, IC and DOC in these extreme environments.

METHODS

Study site. The field experiments were conducted in acidic, stratified Lake 111 and acidic, polymictic Lake Niemegk in 1999. The former is located in Lusatia, a lignite mining area of eastern Germany, the latter was part of the ”Goitsche” mining area near the city of Bitterfeld, before it was flooded with neutral river water. The lakes were chosen for their differences in light climate and DOC concentrations (Table 3-2).

Experiments. Six in situ experiments were carried out. 200 ml bottles were filled with lake water collected from two discrete sampling depths (1 and 2.5 m). The bottles were then incubated at the appropriate sampling depth for 24 h in May 1999 (Lake Niemegk) and August/September 1999 (Lake 111; Table 3-1 and 3-3). Each growth experiment included six treatments: (1) a control with untreated lake water, (2) IC addition as bicarbonated water (final concentration approximately 0.8 mmol L^{-1}), (3) addition of defined DOC plus vitamins (5 mg C L^{-1} each of glucose, glycerol and cysteine was added to a final concentration of 15 mg C L^{-1}, plus vitamins B_1, H, B_{12}), (4) addition of undefined dissolved organic matter as soil extract (0.5-0.8 mg C L^{-1}), (5) addition of dissolved organic phosphorus (glucose-phosphate, 1.6 µmol L^{-1} P final concentration), and (6) dark incubation.

The significance of the treatments on in situ growth rates was tested by a one-tailed *U*-test. Each treatment was replicated five times and each replicate was counted twice (minimum of 600 cells).

Table 3-2. Environmental parameters in the epi- or mixolimnion of the two acidic lakes over the course of the in situ experiments. TIC: Total inorganic carbon, DOC: Dissolved organic carbon, TP: Total phosphorus.

	Lake 111	Lake Niemegk
exposition depth (m)	1; 2.5	1
temperature (°C)	19.4	11.6-14.7
PAR[1] (% I_0)	8-29	3-5
PAR mean[1] (µmol photons $m^{-2}s^{-1}$)	13-100	12-45
TIC (µmol L^{-1})	26-45	74-117
DOC (mg L^{-1})	0.31-0.33	4.00-4.10
SRP (µmol L^{-1})	0.26	1.03
TP (µmol L^{-1})	0.39	5.16 [2]
NH_4^+ (mmol L^{-1})	0.21	0.06
Bacteria[3] (cells L^{-1})	$3.6\text{-}5.1 \cdot 10^8$	$0.8\text{-}9.5 \cdot 10^8$
Chlamydomonas (cells L^{-1})	$1.0\text{-}2.1 \cdot 10^6$	$17\text{-}146 \cdot 10^6$
Ochromonas (cells L^{-1})	$1.8\text{-}5.0 \cdot 10^6$	$0.6\text{-}3.0 \cdot 10^6$
Chlorophyll *a* (µg L^{-1})	0.1-0.6	35-274
Protozoans[4] (cells L^{-1})	$0\text{-}1.1 \cdot 10^3$	$0.06\text{-}6 \cdot 10^4$

[1]- Calculated mean PAR at the incubation depth over the 16 hours with highest irradiance.

[2]- Mainly dissolved phosphorus with 20% SRP.

[3]- Excluding filamentous bacteria.

[4]- In Lake Niemegk, protozoans (mainly ciliates) were removed by sieving (10 µm mesh size). Protozoans in Lake 111 were estimated to be too low to have a significant impact on *Chlamydomonas* population size. Metazoan grazers were extremely low in densities (<10 ind. L^{-1}).

Table 3-3. Impact of nutrient additions and dark incubation on growth rates (d^{-1}) of *Chlamydomonas* in two acidic lakes with 3 experiments in each. Only growth rates of the control and treatment growth rates significantly different from the control are presented (* $p \leq 0.05$, ** $p \leq 0.01$). DOM: dissolved organic matter (soil extract). The calculated μ_{lab} define the growth rates expected from the laboratory results without CO_2 addition at ambient PAR (Fig. 2-2).

Lake	Date	PAR mean[1] (µmol photons $m^{-2}s^{-1}$)	Responsive Treatment	Growth rate (d^{-1})		μ_{lab} (d^{-1})
				Responsive Treatment	Control	
Niemegk	4 May 99	26	Dark	-0.82 ±0.25*	-0.49 ±0.27	0.21
	11 May 99	13	IC	0.16 ±0.14**	-0.10 ±0.09	0.08
	18 May 99	51	Dark	-0.29 ±0.19*	0.15 ±0.20	0.43
111	31 Aug 99	100	DOM	-0.03 ±0.21*	-0.32 ±0.23	0.59
	7 Sep 99	41	DOM	0.35 ±0.07*	0.15 ±0.16	0.36
	14 Sep 99	49	-	-	-0.15 ±0.16	0.42

[1]- Calculated mean PAR in the exposition depth over the 16 hours with highest irradiation.

Photosynthetic available radiation (PAR) was derived from continuous solar radiation measurements at the lakes using a radiometer (Schenk 8101 in Lake Niemegk, Aanderaa 2770 in Lake 111), and vertical PAR attenuation derived from depth profiles (after Koschorreck and Tittel 2002). SRP was determined after Walinga et al. 1989, with pH 2.5 adjusted rinsing water and calibration standards. Algae were fixed with Lugol's solution after addition of 10µl H_2SO_4 (24%) per ml and cell densities were counted in sedimentation chambers.

Laboratory experiments were conducted with *Chlamydomonas* sp. isolated from Lake 111 (strain 11A2). The medium M111 (Bissinger et al. 2000) was used for culturing, which reflected the chemical environment of the Lake 111 water but contained enhanced phosphorus

concentrations (50 µmol L^{-1}). The lake water is naturally buffered by iron-hydroxide (Stumm and Morgan 1979), therefore, in order to test purely autotrophic growth, no organic carbon buffers were added to the medium (Bissinger et al. 2000). However, we measured 0.2-0.3 mg L^{-1} DOC after filtration, due to DOC losses from the filters used when preparing the medium. Given that this DOC was labile and available to the cells, and assuming a consumption rate of 10 % of body C d^{-1}, we calculated that this concentration of DOC could be consumed by the *Chlamydomonas* culture within 3-4 days (equivalent to 2 pg C $cell^{-1}$ d^{-1}). We therefore assumed that the entire DOC potentially available from the medium was consumed during the 5 d pre-culture period employed prior to starting the experiments. Due to zero growth at low irradiances, no further medium needed to be added over the course of the experiment. The pH varied by less than ±0.02 units during the experiments. In the experimental set-up we simulated the special light spectrum in an incubator (Fig. 3-1), supplying light from the bottom (cool neon tubes in combination with 5 and 20W halogen lamps, OSRAM) through a circulating water layer (temperature controlled) and additional colored filters. Light intensity was measured inside the culturing flasks with a quantum sensor (QSL-101, Biospherical) and the light spectrum by means of a spectrophotometer (UW 1800, Li-Cor). Laboratory cultures were kept under a light/dark rhythm of 18/6 hours and pre-adapted to the conditions at least 3 days before an experiment started. In CO_2 enrichment experiments, 5% CO_2 in otherwise normal air was passed through the box, yielding a maximum of 234 µmol IC L^{-1} in the cultures. The cultures were kept semi-continuously according to the turbidostat method and diluted with M111 medium daily to a cell density of $5 \cdot 10^4$ cells ml^{-1}. Cell densities and volumes were measured with a cell counter (CASY 1, Model TT, GAT).

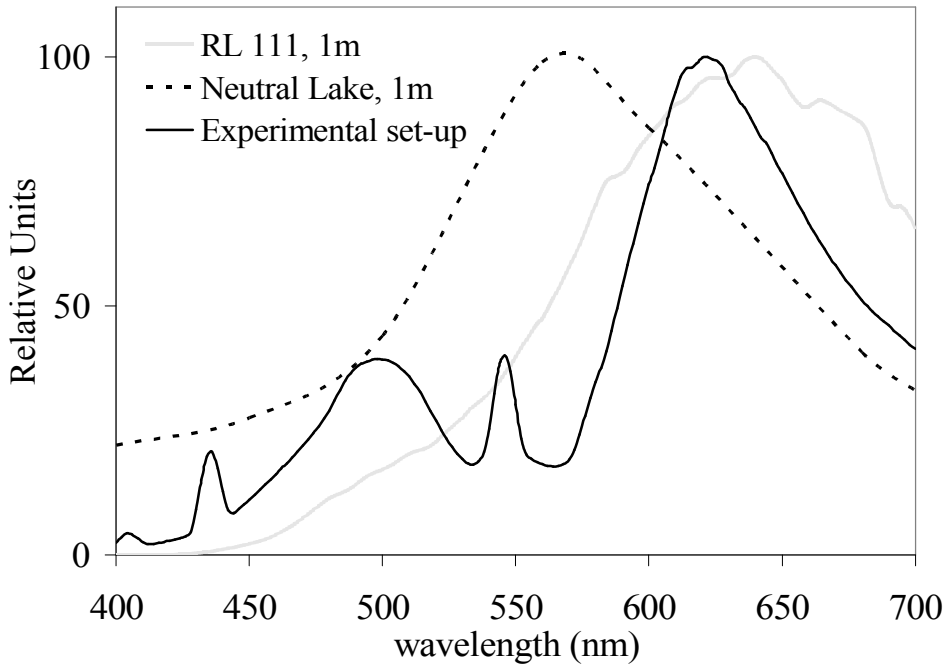


Fig. 3-1. The spectral light distribution in Lake 111 (grey line, 1 m depth) and in the experimental incubator (black line). For comparison, the spectrum of a neutral lake in 1 m depth is inserted (dashed line, Lunzer Untersee, after Sauberer 1962).

To test the potential use of natural DOC for growth, we took lake water from 1 m depth (Lake 111) on November, 13, 2002 and filtered it in two steps using pre-combusted GF/F filters and acid rinsed polycarbonate filters (0.2 µm pore size). Friese et al. (2002) proved the influence of light on the degradation of organic compounds via iron photo-reduction. Therefore, aliquots were exposed for 9 days to 130 µmol photons $m^{-2}s^{-1}$ of red light for 16 hours per day in the incubation box or stored in the dark. We inoculated *Chlamydomonas* in duplicates and monitored its growth for 7 days in the dark by cell enumeration.

Chlorophyll *a* (Chl) was analyzed in duplicates with hot ethanol extraction according to Welschmeyer (1994) with a fluorometer (Turner 10; lamp F4T4$^1/_2$B2 excluding phaeophytin *a*). Carbon was determined by the infrared gas absorption method adapted to acidic conditions with a precision of ≤ 5% at ≥ 0.20 mg IC L^{-1} (after Zippel et al. 2001) in a carbon-Analyzer

("HighTOC+N", Elementar). For POC, duplicate volumes of algal suspensions were filtered (900 °C precombusted QF20 filters, Schleicher and Schuell).

Bacterial carbon was below 1% of algal carbon in most experiments. It did not exceed 2% of algal C in the experiments with addition of glucose.

Growth rates were calculated as $\mu = (\ln N(t) - \ln N_0)/(t - t_0)$, where N_0 was the cell number at time t_0 and $N(t)$ the cell number at time t. The significance of the effect of IC and DOC addition in the laboratory was tested by a two-way ANOVA using SPSS. Squared values were tested for normal distribution. Consecutively, a PostHoc test after Scheffé was performed. Differences in specific Chl contents were tested using a Tukey-test.

RESULTS

Light-dependent growth rates of *Chlamydomonas* exhibited a sigmoid Holling type III shape curve (Fig. 3-2). No significant growth was detectable at photon flux densities below 20 µmol photons m^{-2} s^{-1}. At light intensities ≤10 µmol photons m^{-2} s^{-1}, cell numbers, cell size and cell specific carbon content remained constant for more than 14 days. In complete darkness and without any supplement of organic carbon after up to 4 weeks the cultures were still able to recover in the light and to turn back to normal photosynthetic growth, though pigmentation was reduced in the dark (cf. Table 3-4). Cell size remained constant and cell density declined at most from 5 x 10^6 cells L^{-1} to 1 x 10^6 cells L^{-1} during the 4 weeks, corresponding to a growth rate of –0.06 d^{-1}. No dividing cells and no motion of the cells were detected over the dark period.

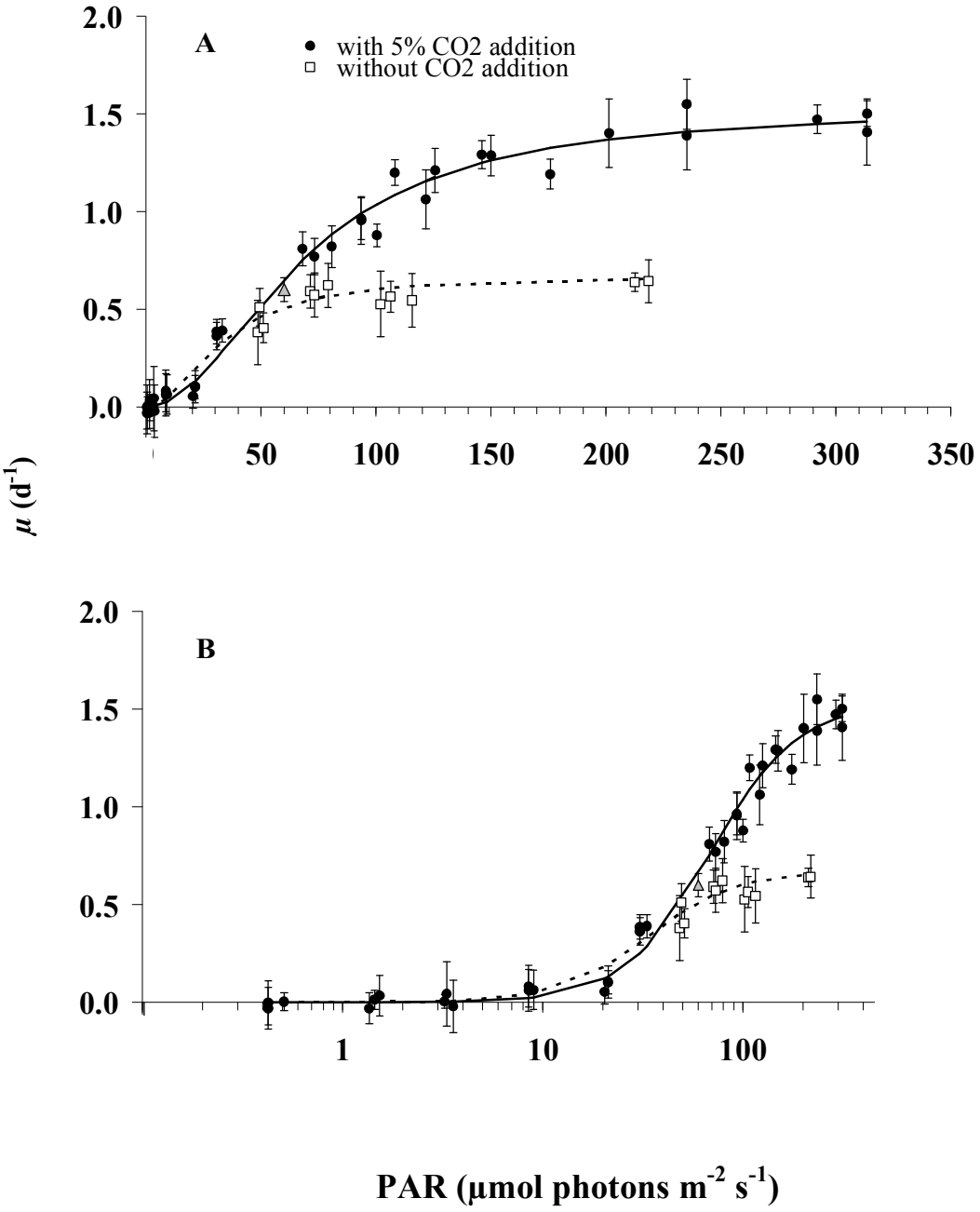


Fig. 3-2. Light dependent growth of *Chlamydomonas* without addition of IC (ca. 13 µmol L^{-1} C) and with enhanced IC (high and medium light intensities: ca. 292 µmol L^{-1} C and 258 µmol L^{-1} C, respectively, light intensities below the compensation point: ca. 92 µmol L^{-1} C) at 20°C. Each point represents the mean of the daily growth rates over a period of 4-10 days. The inserted triangles mark the experiment conducted with filtered lake water. The bars define the standard errors. Curve fitting was performed by inverse regression after Lineweaver-Burk (Ebenhöh 1975); inserted equations define Holling type III curves:

$\mu(+CO_2) = 1.53 \times PAR^2/(PAR^2+69)$; $\mu(-CO_2) = 0.67 \times PAR^2/(PAR^2+34)$.

A) Linear scale;

B) Half-logarithmic scale to illustrate the sigmoid shape of growth rates at low irradiances.

Table 3-4. Specific Chl contents, Chl:C relation, and growth rates of *Chlamydomonas* under the different culture conditions. SE = standard error; SD = standard deviation; D = dark; LL = low light (6-8 µmol photons $m^{-2}s^{-1}$); HL = high light (205-230 µmol photons $m^{-2}s^{-1}$); glu = glucose added; se = soil extract added; n.d. = not determined. Specific Chl content significantly decreased in all cases with glucose addition ($p \leq 0.01$) compared to treatments without organic carbon additions, but was less expressed under HL with CO_2 addition ($p \leq 0.05$).

Culture conditions	CO_2 addition	Specific Chl content (µg mm^{-3} ± SD)	Chl:C (mg/mg)	Growth rate (d^{-1}± SE)
D	no	2.4 ± 0.7	n.d.	0.04 ± 0.21
D + glu	no	<0.1 ± 0.0	0.013	0.26 ± 0.04
LL	yes	18.0 ± 1.2	0.053	0.09 ± 0.05
LL + glu	yes	12.2 ± 0.9	0.033	0.26 ± 0.02
HL	no	9.5 ± 0.7	0.039	0.78 ± 0.09
HL + glu	no	5.4 ± 0.4	0.038	1.05 ± 0.03
HL	yes	8.7 ± 0.6	0.071	1.19 ± 0.08
HL + glu	yes	7.2 ± 0.6	0.025	1.63 ± 0.09
HL + se	yes	7.9 ± 0.8	n.d.	1.26 ± 0.06

Above approximately 20 µmol photons $m^{-2}s^{-1}$ growth rates were significantly positive, which marks the compensation point for cellular growth near this light intensity. When the irradiance exceeded approximately 60 µmol photons $m^{-2}s^{-1}$, growth rates differed between cultures with and without CO_2 enrichment, indicating a switch from light to IC limitation. In Lake 111 at half epilimnion depth (1.5 m), in situ PAR exceeded this light intensity for 11 hours per day in summer (May to September, Table 3-5). The mean PAR in the epilimnion for a 16-hour photoperiod was 138 µmol photons $m^{-2}s^{-1}$ in this depth. In Lake Niemegk, a PAR of 60 µmol photons m^{-2} s^{-1} were exceeded only 7 hours per day at half epilimnetic depth, due to

the lower transparency caused by the high amount of suspended iron hydroxide particle aggregations (Table 3-5). The highest growth rates of *Chlamydomonas* without addition of CO_2 (ca. 13 µmol L^{-1}) were 0.60±0.10 d^{-1} at light intensities ≥ 70 µmol photons m^{-2} s^{-1}. The same growth rates were achieved using sterile filtered lake water instead of medium under in situ epilimnetic conditions (μ=0.60±0.06 d^{-1}, PAR 60 µmol photons m^{-2} s^{-1}, L/D: 16/8 h, Tittel, unpubl.), and fitted in the growth-light-curve (Fig. 3-2). Growth rates under IC enrichment in the laboratory leveled off at approximately 220 µmol photons m^{-2} s^{-1}, which was taken as optimum light intensity in further experiments. Maximum growth rates (μ_{max}) at the highest IC concentrations tested were 1.50 d^{-1}. The carbon content per cell increased with enhanced light supply (Fig. 3-3), but the relative amount of carbon per cell volume remained constant and averaged to 0.23±0.06 pg C μm^{-3}.

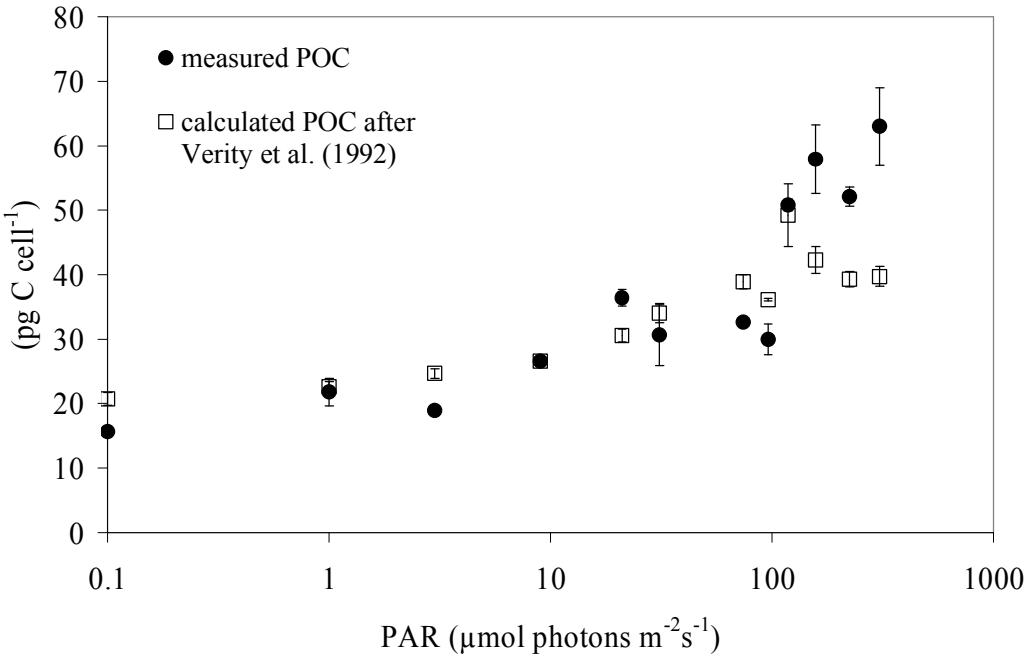


Fig. 3-3. Light dependency of the carbon content of *Chlamydomonas* sp. in growth experiments with addition of 5% CO_2 (black circles), and carbon content calculated from cell volumes after Verity et al. 1992 (white squares).

Table 3-5. Calculated duration of IC limitation of *Chlamydomonas* in mixed epilimnia of different acidic mining lakes (pH 2.6-3.0). We assumed a critical light intensity of 60 µmol photons $m^{-2}s^{-1}$ for a switch from light to IC limitation (see text). z_e: epilimnetic depth; I^*: mean PAR in the epilimnion over a 16 hour photoperiod; t_{IC}: mean daily duration of irradiances >60 µmol photons $m^{-2}s^{-1}$ at half epilimnetic depth (e.g. Lake 117: in 3.8 m). Means of PAR below the surface (I_0) from May to September in one hour intervals were used in the equation $\ln I^* = (\ln I_0 + \ln I_m)/2$ to calculate the mean epilimnion PAR (I^*), where I_m: light intensity at z_e (Reynolds 1997). The seasonal variation of the transmission was low, as it was mainly governed by the iron concentration.

	sampling date	Transmission (m^{-1})	Fe^{3+} (mmol L^{-1})	z_e (m)	IC (µmol L^{-1})	I^* (µmol photons $m^{-2}s^{-1}$)	t_{IC} (hours d^{-1})
Lake 117	19 Aug 99	0.72	0.32	7.6	30	151	11
Lake Sedlitz	18 Aug 99	0.67	0.41	8.1	35	95	10
Lake Skado	18 Aug 99	0.53	1.61	6.8	43	51	5
Lake F	31 Aug 99	0.49	1.36	3.8	48	98	9
Lake 111	7 Sep 99	0.43	2.72	3.2	26	138	11
Lake Niemegk	18 May 99	0.09	1.97	1.5	45	62	7

In the in situ incubation experiments, the variation of the growth rates between replicates was high, despite five replicates per treatment and two pseudoreplicates per replicate, per treatment. The addition of inorganic carbon increased the growth rate in one out of six experiments significantly (Table 3-3). Dark incubation reduced growth relative to the control in only two experiments, indicating non-significant phototrophic growth in the other four experiments. *Chlamydomonas* responded to the addition of DOM (soil extract) in two out of three experiments in Lake 111, which was poor in DOC in the near surface layer compared with DOC-rich Lake Niemegk (Table 3-1) in which an additional supply of organic carbon did not enhance growth rates. We did not observe a growth reaction to the addition of glucose, glycerol and cysteine (+ vitamins), and of organic bound phosphorous within the 24 hour experiments in either lake.

Combining the mean light intensities measured during the in situ experiments and the laboratory derived growth-light relationship without IC enrichment yields significantly higher cellular growth rates than measured population growth rates in situ (Fig. 3-2, μ_{lab} in Table 3-3). This suggests loss factors exist in situ which were not accounted for in the laboratory (e.g. grazing). Microscopic observations revealed potential grazing of *Ochromonas* on *Chlamydomonas*. In the in situ experiments, *Ochromonas* was present and there might have been a grazing influence on the net growth of *Chlamydomonas*. Thus, we measured net rather than gross growth rates in the field data, which fluctuated around zero in most experiments.

To investigate the heterotrophic growth potential of *Chlamydomonas*, soil extract and α-D-glucose were offered separately as organic carbon sources (5 mg C L^{-1} final concentration) in the laboratory. The soil extract had no significant effect on growth. When glucose was added in the dark, the cells exhibited a growth rate of 0.26±0.04 per day (Fig. 3-4). That is, phototrophic growth with optimal light supply and high CO_2 concentration (μ=1.19±0.08 d^{-1}) was approximately 4.5 times higher than heterotrophic growth using glucose only. Under low

CO_2 (approximately in situ concentrations) phototrophic growth was still 2.5 times higher (μ=0.64±0.06 d^{-1}) (Table 3-4).

The addition of glucose in the light enhanced the purely phototrophic growth rates ($p \leq 0.001$) to a similar extent as growth in the dark, even if photosynthesis was saturated by light and CO_2 (Fig. 3-4). The nutrition of DOC thus seemed to supplement growth gained by phototrophy, without stimulation of photosynthesis by internal CO_2 production.

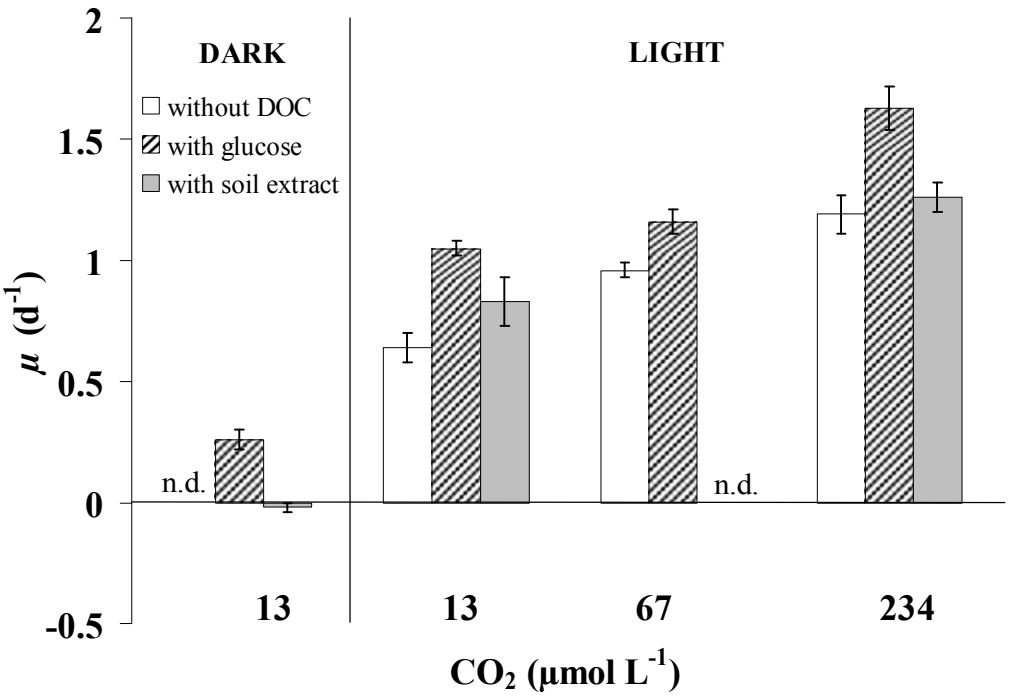


Fig. 3-4. Growth rates of *Chlamydomonas* sp. at different IC concentrations without or with addition of organic carbon sources (final concentration: 5 mg C L^{-1}). Light: 220 µmol photons $m^{-2}s^{-1}$. The columns represent the means of the daily growth rates in semicontinuous cultures over a period of 9-11 days, bars indicate standard errors. n.d.: not determined.

The specific Chl concentrations of *Chlamydomonas* were low in the dark and decreased to almost undetectable levels when glucose was available as organic carbon source (Table 3-4). The chloroplasts of osmotrophically grown cells were fragmented and smaller (Bissinger, unpubl. observ.). Highest Chl concentrations per cell volume were induced under low light

without glucose addition. Under high light conditions, specific Chl was approximately half the amount of that in cells grown under low light conditions. The reduction of the Chl content under high light was most pronounced when CO_2 was low and glucose was added simultaneously. Addition of glucose in all cases significantly decreased the specific Chl content ($p \leq 0.01$), but the effect was lower under saturated photosynthetic conditions ($p \leq 0.05$, Table 3-4).

In the experiments to test the potential of *Chlamydomonas* to use natural DOC in dark growth experiments, the algae realized low but significantly positive dark growth rates in the lake water exposed to the light prior to inoculation (μ= 0.07 ±0.03 d^{-1}, mean ±SE). In contrast, *Chlamydomonas* exhibited a slightly negative dark growth in non-pre-illuminated water (μ= -0.10 ±0.06 d^{-1}). The concentration of Fe^{2+} increased in the illuminated water compared to the control (41 µmol L^{-1} and 25 µmol L^{-1}, respectively).

DISCUSSION

We obtained internally consistent laboratory results providing unequivocal evidence allowing us to accept the hypotheses (H-1), (H-2) and (H-4) and reject (H-3). Interpretation of field results turned out to be more complex as some factors could not be controlled in situ.

Low IC concentrations in the epilimnion limit photosynthesis (H-1).

Evidence for algal growth limitation by IC in situ have to date been restricted to water bodies with high pH. IC limitation at pH>8 is the effect of low CO_2 concentrations under alkaline conditions and has been shown in eutrophic freshwater systems in bloom situations (Klemer et al. 1982; Hein 1997). In marine system, the large fluctuations in light intensity by vertical mixing are assumed to cause the highly variable photosynthetic fixation of carbon (Riebesell 2000), which some marine diatoms, incapable of using bicarbonate as IC source, circumvent by developing a C_4 pathway to avoid CO_2 limitation (Reinfelder et al. 2000). In acidic lakes,

in situ IC limitation could never be proven, but was assumed by most authors and held responsible for, for example, low biomass specific primary production (Lessmann et al. 1999) or enhanced respiration rates (Turner et al. 1991). Previous investigations on IC limitation evaluated the growth characteristics of the organisms under otherwise optimal laboratory conditions (e.g. Goldman and Graham 1981; Olaveson and Stokes 1989) and increasingly focused on processes in cell metabolism (e.g. Moroney 2001), which complicates extrapolation to in situ conditions. We aimed to simulate the specific chemical and light conditions of our investigated lake in culture experiments. A comparison of growth rates of *Chlamydomonas* in the artificial medium and in lake water under otherwise identical conditions revealed similar results. This enabled us to assume that our laboratory results were relevant for field conditions.

In our laboratory experiments, CO_2 supplementation enhanced the photosynthetic growth above a light intensity of 60 µmol photons m^{-2} s^{-1} and doubled it at the start of light saturation (ca. 150 µmol photons m^{-2} s^{-1}). In order to be able to apply the results to field situations, we calculated the duration of IC limitation over the photoperiod (t_{IC}; Table 3-5). In situ, light intensities exceeded 60 µmol photons m^{-2} s^{-1} down to a depth of 1.5 m (half of the epilimnetic depth) during most of the day in Lake 111 from May to September, suggesting that light limitation is unlikely in this upper stratum and time period and CO_2 limitation therefore predominates (Table 3-5). IC limitation is also evident in two other acidic lakes (Lake 117, Lake Sedlitz) (Table 3-5), assuming that the chlamydomonads found in these lakes exhibit comparable growth characteristics as the strain investigated here. Hence, our results suggest that on average IC limitation was indeed relevant in the upper stratum of the water column during the growing season in acidic lakes, whereas light limitation was presumably the dominant growth regulating factor during less stratified periods and at greater depths. In terms of individual algal cells, rapid alternations between CO_2 and light limitation may occur

throughout the epilimnion given the high variability in irradiance and residence depth of individual algal cells during the day. In contrast to other nutrients, light and CO_2 can limit instantaneous cell growth, but not the evolving biomass, as they are continuously supplied. Thus, loss processes must account for the low biomass in many of these lakes, rather than the extreme chemical conditions or limitation by CO_2.

In our in situ experiments, IC addition affected growth rates in only one out of six replicates within 24 h. This low responsiveness may be explained by the light intensities at the incubation depths, which were in all cases below or around the critical light intensity of 60 µmol photons m^{-2} s^{-1} (Table 3-3). According to the observed high standard deviations between the in situ replicates, measuring an effect would require higher light intensities. Additionally, in situ IC concentrations were higher than in the cultures without IC addition (26-117 and 13 µmol L^{-1} C, respectively).

In conclusion, (H-1) was confirmed by our results; the phototrophic growth of the dominant phytoplankton species was IC limited under conditions typical for the upper strata of the epilimnion in acidic lakes.

Mixotrophy leads to higher growth rates under IC limitation (H-2).

Our results confirmed (H-2) as *Chlamydomonas* enhanced its growth significantly through osmotrophic nutrition when organic carbon was added, independent of photosynthesis being saturated or limited by light or inorganic carbon (Fig. 3-4 and Table 3-4). The strength of the reaction to DOC addition depended on the organic carbon source. In the laboratory cultures, pure glucose yielded the most pronounced enhancement of growth rates under heterotrophic and mixotrophic conditions, whereas the addition of terrestrial soil extract (same amount of DOC) had no significant effect. In situ, the addition of soil extract from the lake shore resulted in significantly increased growth rates in two out of three treatments in Lake 111,

where natural DOC concentrations were low (<0.5 mg L^{-1}, Table 3-1). In Lake Niemegk, DOC concentrations were high in the whole water column (Table 3-1), and the in situ DOC additions did not result in a further increase of growth rates. The in situ addition of a mixture of glucose, glycerol and cysteine did not effect growth within the 24 h incubation period. From batch cultures it is well known that the cells need some time to adapt to altered environmental conditions after inoculation, due to the formation of specific enzymes for metabolism (Laliberté and Noüe 1993). Presumably the incubation time was too short for the natural flagellates to react to the artificial organic carbon sources. Similarly, glucose additions enhanced growth in a laboratory strain of *Euglena mutabilis* at pH 3.0 but not in a field strain (Olaveson and Stokes 1989).

Active uptake and metabolization of natural DOC is generally difficult to show. Tracer experiments showing the uptake of single organic substances commonly suffer from a lack of knowledge of how representative the compound is for the bulk of dissolved organic compounds. Furthermore, they do not provide information as to whether this compound is metabolized and used for cellular growth (Vincent and Goldman 1980). The dark growth of *Chlamydomonas* in filtered lake water gave a clear indication that natural DOC from Lake 111 is taken up and metabolized. The experiments also indicate that light mediated processes, probably photoreduction of Fe^{3+} to Fe^{2+} and subsequent DOC degradation (Friese et al. 2002), rendered the DOC available for consumption by algae.

The specific Chl content was significantly reduced when organic carbon was available (Table 3-4). Lewitus and Kana (1994) observed species-specific pigment responses in phytoplankton in glucose treatments which ranged from total loss, through reduction and no change, to increase of Chl. In our experiments, the decrease was less expressed with increasing phototrophic growth, i.e. lower under high light conditions and CO_2 supply ($p < 0.05$, 17 % reduction in specific Chl), than under CO_2 limitation ($p < 0.01$, 43 % reduction). Thus,

mixotrophic nutrition was likely accompanied by a loss of photosynthetic capacity, but resulted in a net increase of cellular growth.

The catabolism of exogenous organic carbon provides intracellular CO_2 which stimulates photosynthesis (H-3).

Additional DOC uptake may stimulate growth under IC limitation a) by purely heterotrophic use of DOC in addition to phototrophic growth, or b) by photosynthetic refixation of released CO_2. When CO_2 is added under high light conditions the difference between autotrophic and mixotrophic growth is expected to remain constant if a) is relevant, or to decrease if b) holds. To distinguish between these alternatives we measured autotrophic and mixotrophic growth rates of *Chlamydomonas* under various conditions and found that the difference between autotrophic and mixotrophic growth was independent of the CO_2 concentration (Fig. 3-4, Table 3-4). Thus, (H-3) was rejected. *Chlamydomonas* can use glucose as an organic carbon source in the dark (heterotrophically) and as a supplementary carbon source during phototrophic growth. Other experiments with the same alga support this finding, as ^{14}C marked glucose was completely incorporated into biomass in the dark, and no respiration to $^{14}CO_2$ was detected (N. Kamjunke, University of Potsdam, unpubl.).

The adverse environmental conditions in the acidic lakes increase metabolic costs and reduce maximum growth (H-4).

Our *Chlamydomonas* strain exhibited clearly reduced maximum phototrophic growth rates compared to the range of growth rates (2.3-2.9 d^{-1}) quoted by Reynolds (1984) for algae of comparable size in non-acidic conditions. The light intensity required to achieve positive growth significantly different from zero for our *Chlamydomonas* strain was high in our experiments (10-20 µmol photons $m^{-2}s^{-1}$) indicating high light demands for positive growth in

situ. In many studies about growth-irradiance-relationships, data points are extrapolated to the compensation light intensity. Gervais (1997) investigated growth in *Cryptomonas* at very low light intensities and found a compensation point close to 0 µmol photons m^{-2} s^{-1}. It should be noted that compensation light intensities given in these and other studies correspond to zero cellular growth which can, however, be assumed to be close to the irradiance needed for positive growth. The initial increase of our light-growth curve was shifted to higher light intensities. We interpret these findings as being the result of enhanced metabolic costs for growth in this chemically extreme environment. Therefore, (H-4) was supported by high light demands for positive growth and a low maximum growth rate. The hypothesis was also verified with CO_2 addition which implied that other factors like the high H^+ or metal concentrations may also enhance the metabolic costs.

Other small green algae grown under acidic conditions, e.g. *C. acidophila* at pH 5.0 (Erlbaum Cassin 1974) or *Dunaliella acidophila* at pH 1.0 (Gimmler and Weis 1992), exhibited comparable or even lower maximum phototrophic growth rates (0.40-0.70 and 0.09, respectively) than our culture (1.5 with CO_2 addition). A new isolated acidophilic xantophyte, *Chloridella* sp., from a volcanic lake achieved phototrophic growth rates of 0.41 d^{-1} with CO_2 saturation (Diaz and Maberly, submitted). Our *Chlamydomonas* strain exhibited its highest growth rates under mixotrophic conditions (1.66 with CO_2 and glucose addition) similar to *C. acidophila* grown with added glucose addition at pH 5.0 (mixotroph: 0.93, osmotroph: 0.20) (Erlbaum Cassin 1974), and *C. humicola* grown with acetate addition at pH 7.2 (mixotroph: 1.66, osmotroph: 0.78) (Laliberté and Noüe 1993). In *Euglena mutabilis*, mixotrophy did not enhance growth above that of purely phototrophic CO_2-aerated cultures at pH 3.0, but glucose was also the best organic substrate (Olaveson and Stokes 1989).

The light dependent growth of our *Chlamydomonas* strain followed a Holling type III curve exhibiting a sigmoid shape. This implies that losses must be variable, probably decreasing

with decreasing growth rates. We did not find reference to any other study with a similar lagged initial slope. Typically, phototrophically grown algae exhibit negative growth rates below the compensation point and a strong responsiveness to increasing light intensities above it (e.g. Langdon 1987; Coesel & Wardenaar 1994).

The remarkable starvation resistance under low light conditions which we observed in our *Chlamydomonas* strain suggests the induction of a metabolically reduced state when energy supplies are low, comparable to a resting/dormant stage. We interpret the special characteristics of the growth-light-curve at low light intensities as an adaptation to the extreme conditions.

In non-acidic lakes, photosynthetic growth is often limited by mineral nutrients in illuminated lower strata. The metabolic use of DOC by algae gained importance when cellular growth rates were limited by light in deeper stratified layers (Lewitius and Kana 1994). In Lake 111, the chlamydomonads evidently used their osmotrophic potential in a range of environmental conditions including those in the illuminated epilimnion. Our interpretation is that low IC concentrations in the epilimnion limit photosynthesis and, analogous to algae in the deeper layers of non-acidic lakes, this results in an energy and/or carbon limitation of growth and favors the metabolic use of DOC. High light requirements for photosynthetic growth, attributed to metabolic costs, further render mixotrophy relevant even in the epilimnion.

Chlamydomonas is commonly regarded as an *r*-strategist (Reynolds 1997) but our strain exhibited numerous characteristics generally attributed to *K*-strategists: we calculated low in situ growth rates and our strain was obviously able to reduce metabolic costs at low light levels. *Chlamydomonas* densities fluctuated relatively little throughout the growing season (Wollmann et al. 2000; Tittel unpubl.). Furthermore, *Chlamydomonas* dominance was primarily based on its competitive abilities rather than on the physiological exclusion of other

phototrophic species by the adverse environmental conditions. We isolated other mainly phototrophic algal species from the water of Lake 111 and cultivated them in the acidic medium but encountered them only sporadically in our samples. Low maximum growth rates, avoidance of losses, low fluctuations of population sizes, as well as high competitive abilities, are characteristic of *K*-strategists (Sommer 1981) successfully colonizing abiotic stable environments. The acidic lakes do represent stable environments, due to stable stratification and low variability in, e.g. nutrients, light attenuation, acidity and ion concentrations.

ACKNOWLEDGEMENTS

This research was supported by the German Ministry of Education and Research (BMBF). We thank Sabine Donath, Ines Hübner and Cornelia Hesse for assistance in the laboratory. Gregor Fussmann provided support with statistics and Ulrich Sommer, Walter Geller, Guntram Weithoff and Elanor Bell provided helpful comments on the manuscript.

4 PHOTOSYNTHETIC RATE

Photosynthetic light-response curves in *Chlamydomonas* sp. with respect to light, CO_2 and dissolved organic carbon

Vera Bissinger

ABSTRACT

The photosynthetic capacity of *Chlamydomonas* sp., isolated from acidic mining Lake 111 (pH 2.7), was measured by recording photosynthetic light-response (*P/I*) curves. This was the first investigation of *P/I* characteristics in this mixotrophic alga from an extreme acidic mining lake carried out in order to study the photosynthetic response of *Chlamydomonas* sp. to carbon addition under different light conditions. Algal cultures were grown at 20°C under high light (HL, 220 µmol photons m^{-2} s^{-1}) and low light (LL, 10 µmol photons m^{-2} s^{-1}) conditions and their photoadaptive photosynthetic responses were compared. The influence of inorganic carbon (by CO_2 addition) and organic carbon (by glucose addition) on photosynthesis was tested in different treatments under the two light intensities. When grown under HL, dark respiration rates (R_d) and maximum photosynthetic oxygen production rates (P_m) were significantly higher compared to LL (p<0.001). The addition of CO_2 decreased R_d and enhanced the net amount of oxygen production, i. e. the difference between P_m and R_d. This supports our former findings that low epilimnetic inorganic carbon concentrations may reduce the autotrophic growth potential of *Chlamydomonas* sp. The highest values of P_m were recorded under mixotrophic conditions (i.e. with glucose addition) with surplus CO_2 at HL.

Under LL, reflecting in situ conditions at the deep chlorophyll maximum (DCM) persisting in many acidic mining lakes, supplementing the cultures with CO_2 or glucose did not enhance P_m. Although the cells seemed to increase their photosynthetic potential by enhanced cellular pigment contents, light would appear to be the predominant limitation factor for photosynthesis, maintenance and growth of *Chlamydomonas* sp. at the DCM.

INTRODUCTION

Photosynthetic light-response (*P/I*) curves can be used to characterize photoacclimation in algae, providing useful information about their photosynthetic capacity (Gilbert et al. 2000, Polle et al. 2000). *P/I* curves enable a basic ecophysiological characterization of natural and cultivated algal populations with respect to their light demands and the adaptation state of the cells. Generally, the results from growth-irradiance (μ-*I*) curves provide more reliable predictions of the in situ responses of an algal population to altered growth conditions in its natural environment than *P/I* measurements because they are conducted over a longer time period. However, potential photosynthesis, as evaluated by *P/I* curves, can provide interesting additional information about in situ productivity of algae, especially as some algae express their maximum biovolume in deeper strata (Adler et al. 2000), close to their compensation irradiance for growth. In several acidic mining lakes, deep chlorophyll maxima have been found to be dominated by *Chlamydomonas* sp. (see Chapter 6, Nixdorf et al. 1998) generating interest in the photosynthetic adaptations of this alga to low light. This adaptation can be studied by measuring *P/I* curves. In contrast to μ*/I* curves or standard ^{14}C uptake measurements, *P/I* curves also provide dark respiration rates which enables a better differentiation between phototrophic and heterotrophic nutrition of the mixotrophs.

This Chapter provides the first description of the photosynthetic characteristics in *Chlamydomonas* sp., isolated from the extremely acidic Lake 111 (Chapter 2), in relation to

light acclimation, CO_2 and dissolved organic carbon (DOC) concentrations, using *P/I* curves. Photosynthesis under contrasting lake conditions is compared by using cultures adapted to epilimnetic and hypolimnetic light conditions. The optimum light intensity for maximum growth obtained from growth experiments (Chapter 3) was employed as the high light (HL) level (epilimnetic conditions) and the light intensity at the compensation irradiance for growth (Chapter 3) as the low light condition (LL; hypolimnion at the deep chlorophyll maximum=DCM). In addition, *P-I* curves were measured under different CO_2 and glucose concentrations since the μ-*I* curves of *Chlamydomonas* sp. were altered by these factors (cf. Chapter 3). The responses of *Chlamydomonas* as indicated by its *P-I* curves to CO_2 and glucose alternations determine the autotrophic and osmotrophic growth potential of the alga and may help to explain the observed growth dependency on these parameters (cf. Chapter 3).

METHODS

Chlamydomonas sp. isolated from Lake 111 was pre-cultured for at least 14 days in medium M111 (Bissinger et al. 2000, Chapter 2) under different conditions: at high light (HL = 220 µmol photons m^{-2} s^{-1}, epilimnetic light spectrum see Fig. 3-1), at low light (LL = 10 µmol photons m^{-2} s^{-1}, hypolimnetic light spectrum in the DCM; see Koschorreck and Tittel 2002), with and without supplementation of α-D-glucose (DOC, 20 mg C L^{-1}), and with or without addition of CO_2 (final concentration in the cultures ca. 230 and 13 µmol C L^{-1}, respectively). It has to be mentioned that the light intensity of the LL treatment was still higher than in the DCM (DCM: ca. 3 µmol photons m^{-2} s^{-1}) due to the set-up, but still far below the compensation point (Chapter 3).The cells were grown in 300 ml Erlenmeyer glass flasks at 20°C, under a 16/8 h light/dark cycle. Chlorophyll *a* (Chl) was determined by extraction in 80% hot ethanol solution according to Welschmeyer (1994) with a fluorometer (Turner 10).

Rates of photosynthetic oxygen evolution (positive values) and respiratory oxygen consumption (negative values) were measured using the light pipette system PLD 2 (Topgallant LLC, Salt Lake City, USA). Samples were incubated with increasing actinic light intensities (white light, 0 to 1800 µmol photons m^{-2} s^{-1}; 20 min), each light intensity lasting for two minutes on average, lower light intensities lasting longer. The rate of O_2 production per unit volume of *Chlamydomonas* sp. culture (µmol ml^{-1} h^{-1}) was determined with a Clark type micro-oxygen electrode (Microelectrodes Inc., USA) calibrated in water samples equilibrated with air (100% O_2 saturation) and containing sodium sulfate (0% O_2 saturation). Temperature was controlled by circulating water regulated by a thermostat (Julabo). For each treatment studied the measurements were repeated three times with exchanged algal solution. The data obtained were fitted to the relationship:

$$P = P_m(1 - e^{-\alpha I/Pm}) + I\beta + R_d \qquad \text{(Equation 1; Walsby et al. 2001)}$$

in which P is the oxygen production, P_m is the light-saturated rate of gross oxygen production (= photosynthetic capacity), R_d is the respiration rate in the dark, α is the initial slope of a straight line fitted to the *P/I* curve at low irradiance and β represents the slope at high irradiances, describing photoinhibition. The values of the coefficients P_m, α and β were determined by the least squares method, using the Solver software in Excel (after Walsby 2001). I_k is defined as the irradiance at which a straight line fitted to the *P/I* curve at low irradiances intercepts with P_m. It can be used to differentiate between LL and HL acclimation (Kohl and Nicklisch 1988) and was computed as $I_k=P_m/\alpha$. From the Chl concentration in the algal suspensions (mg ml^{-1}) the Chl specific rates of O_2 production were calculated (mmol O_2 $gChl^{-1}$ h^{-1}) to normalize the *P-I* curves, because Chl may vary among cultures with different pre-incubation conditions (see Chapter 3). Cultures were dark adapted for ca. 30 minutes prior to measuring dark respiratory O_2 uptake at the beginning of the measurement with the light pipette.

Differences in photosynthetic rates between treatments with low and high CO_2 and DOC concentrations were tested by a two-way ANOVA.

RESULTS

The photosynthetic parameters of the *P/I* curves of *Chlamydomonas* sp. cultures strongly depended on culture conditions. High light (HL) adapted cells expressed significant higher dark respiration rates (R_d) ($p<0.001$) and maximal oxygen production (P_m) ($p<0.001$) than those grown under low light (LL) (Table 4-1). R_d decreased under CO_2 supplementation in the HL cultures ($p<0.01$) and glucose addition enhanced P_m ($p<0.05$) (Table 4-1). In the LL adapted cultures, both R_d and P_m were not significantly different between the individual LL treatments. Photosynthetic oxygen production of cells grown under LL, low CO_2 concentration and without organic carbon source was below detection and only R_d could be estimated as represented in Table 4-1. Unexpectedly, R_d yielded more than 70% of the total (gross) oxygen production (= P_m) in the cultures without CO_2 addition and acclimated to HL, whereas it was around or below 30% in all other treatments (Fig. 4-1). α decreased with CO_2 addition at HL and I_k consequently increased. At LL, no reaction of these parameters to the carbon treatments was observed (Table 4-1). Photoinhibition, indicated by non-zero values of the parameter β (the slope at high actinic light intensities), was not measured in any *P/I* curve.

Table 4-1. Characteristic *P/I* curve parameters of *Chlamydomonas* sp. Comparison between high light (HL, 220 µmol photons m^{-2} s^{-1}) and low light (LL, 10 µmol photons m^{-2} s^{-1}) acclimated cultures and different treatments with or without supplementation of CO_2 and glucose (glu). R_d = dark respiration, P_m = photosynthetic capacity, α = initial slope of the *P/I* curve and $I_k = P_m/\alpha$. The parameters were obtained by fitting the mean of 3 replicate measurements to Equation 1. b.d.: oxygen production below detection.

	R_d (mmol O_2 $gChl^{-1}$ h^{-1})	P_m (mmol O_2 $gChl^{-1}$ h^{-1})	α	I_k (µmol photons m^{-2} s^{-1})
HL +CO_2 +glu	-197	770	2.7	288
HL -CO_2 +glu	-485	680	4.1	167
HL +CO_2 -glu	-141	504	2.0	257
HL -CO_2 -glu	-553	689	4.9	139
LL +CO_2 +glu	-48	326	2.4	135
LL -CO_2 +glu	-103	388	3.3	118
LL +CO_2 -glu	-96	309	3.2	96
LL -CO_2 -glu	*-88*	b.d.	b.d.	b.d.

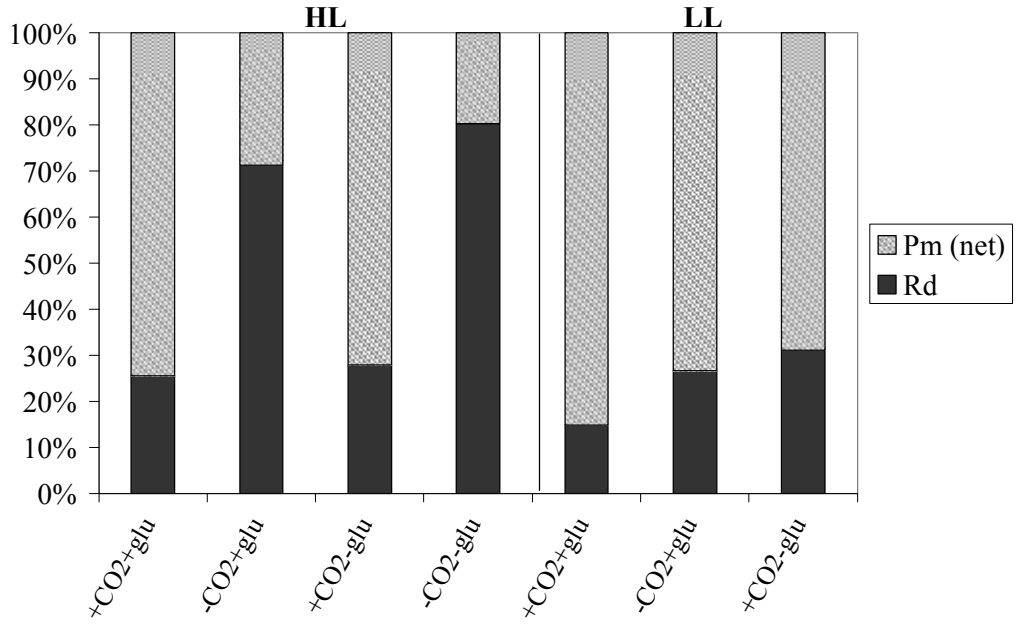


Fig. 4-1. Fraction of dark respiration (R_d) to gross oxygen production (100%) in the different treatments. HL: high light adapted cultures (220 µmol photons m^{-2} s^{-1}), LL: low light adapted cultures (10 µmol photons m^{-2} s^{-1}).

DISCUSSION

The photosynthetic characteristics of algal cells measured in photosynthetic light-response curves, change as the cells adapt to the light intensity in their environment (Gallagher et al. 1984). Since the experimental conditions of our growth (Chapter 3) and photosynthesis experiments were identical, the effects of inorganic and organic carbon addition under the different light intensities are directly comparable. Thus, we can confer our data concerning the short-term consequences of these factors on the photosynthetic performance on the results obtained from the long-term reactions of realized growth rates.

The measurement of *P/I* curves in *Chlamydomonas* sp. cultures grown under high light (HL) and low light (LL) conditions offered insight into the photosynthetic parameters of these mixotrophic organisms. The largest effect of light acclimation presented here was a higher dark respiration rate (R_d) at HL compared to LL. Respiration activity generally describes the conversion of the primary products of photosynthesis (carbohydrates) to new cell material (e.g. protein). A higher R_d at higher light acclimation is common in many other algal species (e.g. Falkowski et al. 1985). *Chlamydomonas reinhardtii* at pH 7 exhibited an R_d of -79 mmol O_2 $gChl^{-1}$ h^{-1} at light saturation and 5% CO_2 (Heifetz et al. 2000). This value was lower than that of the comparable HL+CO_2-glu culture (-141 mmol O_2 $gChl^{-1}$ h^{-1}, Table 4-1) in this study which may be attributable to the increased maintenance costs of *Chlamydomonas* sp. sustained when living under the extreme environmental conditions (see below, cf. Chapter 3). The supplementation of the cultures with CO_2 decreased R_d at HL (Table 4-1). The lower R_d values caused a significantly higher net oxygen production (P_m-R_d) with addition of CO_2 under HL (Fig. 4-1), pointing to the effect of inorganic carbon limitation on photosynthetic processes under these irradiances and at low pH. The significantly higher I_k values at HL +CO_2 support these results, as an increased I_k often indicates a more efficient use of high irradiances (Henley 1993). Under low CO_2 concentrations and HL, net oxygen production

was even lower than in the corresponding LL treatment. Our investigations on long-term growth rates showed the moderate growth potential of the cells under these conditions (HL, low CO_2, without glucose: μ= 0.78±0.09 d^{-1}, Chapter 3). The increased R_d at low CO_2 concentrations might be explained by a need to reduce the intra-cellular ratio between O_2 and CO_2 in the vicinity of Rubisco, which might be achieved by a carbon concentrating mechanism that increases internal CO_2 concentration (Moroney and Mason 1991, Badger et al. 1998, Tortell 2000). The specific problem for phototrophic organisms under the extremely acidic conditions is that no HCO_3^- is available. HCO_3^- generally represents an unlimiting inorganic carbon source under circumneutral conditions, but in the acidic mining lakes IC is only available as CO_2 (Stumm and Morgan 1970). This may imply that the activity of the mitochondria is enlarged resulting in a higher glycolysis and higher values of R_d (Villarejo et al. 1995).

The highest P_m under HL acclimation was determined in mixotrophic cultures treated with both enhanced CO_2 and glucose concentrations. Unexpectedly, glucose addition under HL did not enhance R_d compared to treatments without glucose. These results are not immediately explicable since glucose respiration should consume O_2. If the additional use of CO_2 internally metabolized by glucose respiration (Villarejo et al. 1995) would have caused the increased P_m, this effect should be even higher without CO_2 supply. As this was not the case (Table 4-1), this outcome could not be satisfactionally clarified in this study.

The *P/I* curves from LL adapted cultures closely reflect the status of the cells under in situ hypolimnetic conditions (in the DCM, see Chapter 6). Light is considered the predominate limitation factor for growth of *Chlamydomonas* sp. at this depth. Therefore, CO_2 supplementation was not expected to contribute to higher photosynthetic rates. Indeed, *Chlamydomonas* sp. did not regulate its P_m in response to CO_2 and/or DOC addition at LL (Fig. 4-1).

Under LL conditions, P_m was lower than at HL. However, as values of R_d were also lower, the overall net oxygen production (P_m+R_d; with negative R_d) resulted in comparable values as under HL (+CO_2).

Comparing *Chlamydomonas* sp. from acidic conditions (HL+CO_2-glu) with results obtained from *C. reinhardtii* at pH 7 (saturating light, +5% CO_2, 25°C) (Heifetz et al. 2000), the net P_m was similar (363 and 345 mmol O_2 $gChl^{-1}$ h^{-1}, respectively). These results also indicate that photosynthesis of *Chlamydomonas* sp. at HL was restricted by the low CO_2 concentrations (net P_m under HL-CO_2-glu: 136 mmol O_2 $gChl^{-1}$ h^{-1}). It would appear that in situ, *Chlamydomonas* cells exhibit a higher photosynthetic capacity (higher P_m) in the epilimnion than in the DCM, but improve their photosynthetic potential under DCM conditions, owing to increased specific Chl *a* contents in low light (cf. Chapter 3).

Without CO_2 or glucose supplementation under LL, oxygen production was below the detection limit (Table 4-1). The formerly observed zero-growth of light limited cells (see Chapter 3: loss of motion and cell division but high starvation resistance) is thus confirmed by the strong reduction of oxygen production. The cells still exhibited dark respiration, indicating metabolic costs for cell maintenance under these conditions. These costs cannot be quantified by measurement of growth rates (Chapter 3).

In other investigations on phototrophs, α increased or remained constant from HL to LL (Kohl and Nicklisch 1988, Walsby et al. 2001). The *Chlamydomonas* strain from the acidic mining lake showed no increase of α under LL. The reason may be the higher amount of Chl per unit cell volume under LL acclimation (see Chapter 3) and adjusting α by relating the oxygen production to Chl concentration. As a result, α may be similar (Kohl and Nicklisch 1988). As the shape of the *P/I* curves closely depends on the light conditions, the importance of adjusting experimental conditions to the spectral quality which the organisms face in their natural environment and as applied in this study, is thus strongly recommended for further

investigations. The absence of photoinhibition (negative β) under the investigated actinic light intensities (max. 1800 µmol photons m^{-2} s^{-1}) was in agreement with other green algae. Therefore, the highest light intensity employed in our experiments was insufficient to cause photoinhibition.

The presented results exhibited that the *P/I* characteristics of mixotrophic *Chlamydomonas*. sp. from an extremely acidic mining lake varied with light acclimation. The results form another strong indication of CO_2 limiting conditions in the epilimnion of Lake 111 (epilimnetic IC concentrations: 26-45 µmol L^{-1}). Moreover, the results support the supposition that mixotrophy could improve photosynthetic oxygen production under HL, in the same manner that it enhanced growth rate (Chapter 3), but only when there is an adequate supply of CO_2. However, the low epilimnetic DOC concentrations in Lake 111 (0.32 mg L^{-1}), restrict the potential for mixotrophic growth in situ in upper strata.

Conversely, in the hypolimnion of Lake 111 at the DCM, higher concentrations of CO_2 and DOC are available and light is the predominant limitation factor for photosynthesis and growth (cf. Chapter 3). With suitable organic carbon sources, *Chlamydomonas* is likely to use its mixotrophic potential for maintenance and low growth in light limited strata. Future investigations describing the photosynthetic physiological adaptations and specific enzyme activities in this alga, in response to different light intensities and carbon concentrations, will further elucidate the specific adaptations of *Chlamydomonas* sp. from the acidic environments.

ACKNOWLEDGEMENTS

I would like to thank Dr. Elly Spijkerman and Antje Gerloff for their introduction into the secrets of the light pipette and especially the calculation and discussion of the *P/I* curves. Cornelia Hesse measured the chlorophyll. Prof. Ursula Gaedke and Dr. Elly Spijkerman offered helpful and supplementary comments on the manuscript.

5 NUTRIENT DEPLETION EXPERIMENTS

The effect of phosphorus (P) availability on alkaline phosphatase activity (APA) in *Chlamydomonas* sp.

Vera Bissinger

ABSTRACT

According to previous investigations, phytoplankton growth in acidic mining Lake 111 is primarily limited by inorganic carbon (IC), but inorganic phosphorus (P_i) is also hypothesized to contribute to low cell densities in epilimnetic layers. Existing evidence of P limitation is contradictory. In this study, I tested the induction of extracellular alkaline phosphatase activity (APA) in laboratory cultures and in situ, to investigate availability of P in acidic mining lake phytoplankton. The determination of APA in single *Chlamydomonas* cells by the ELF-method (Gonzàlez-Gil 1998) was applied for the first time in acid mining lake samples. APA was clearly induced in all field samples indicating P depletion of individual cells, but was not induced in P-enriched cultures. Growth rates were related to the internal P content of the cells. The realized cell densities in situ in the epilimnion were an order of magnitude lower than the carrying capacity (calculated by dividing the ambient SRP by the individual P content of the cells). This may partially be attributed to i) top-down control (Chapter 6), ii) restricted SRP availability, or iii) an increased P demand under CO_2 limitation.

INTRODUCTION

Inorganic carbon (IC) was hypothesized to limit photosynthetic growth in the acidic mining lakes and experiments performed in situ and under laboratory conditions strongly support an IC limitation in epilimnetic phytoplankton during summer months (Chapters 3 and 4). However, phosphorus (P) has also been suggested as growth limitation factor in the mining lakes (Nixdorf et al. 1998, Lessmann et al. 1999, Beulker et al. 2002). To date, the results from investigations of the potential for P limitation in the mining lakes have been contradictory. Incubating lake water in bottles in situ with dissolved organic P supplements had no effect on growth over 24 h (Chapter 3). Likewise, under laboratory conditions, the measured growth rates of *Chlamydomonas* sp. cultured in unenriched, filtered lake water were identical to those in P-enriched medium after several days in semicontinuous culturing (Chapter 3). Soluble reactive P (SRP) concentrations in the epilimnion of Lake 111 (ca. 7 µg L^{-1} without a pronounced vertical gradient) do not indicate P limitation being above the threshold value commonly associated with P limitation in most algal species in circumneutral lakes (Sas 1989). Moreover, the in situ SRP concentrations suggest a higher potential algal standing stock should be supported in the epilimnion than is actually observed. The high aluminum concentrations in many acidic mining lakes (up to 40 mg L^{-1}) can cause complexation with P (Joseph et al. 1995) and influence P availability and metabolism (Olsson and Petterson 1993) and in this way may account in part for the low standing stock observed. In addition, in saline lakes, high sulfate concentrations were assumed to contribute to lower availability of P (Waiser and Robarts 1995). This might also be the case in the mining lakes where sulfate concentrations can reach 4000 mg L^{-1}. The low cell densities of *Chlamydomonas* sp. in the epilimnion of Lake 111 cannot be explained by IC limitation. A potential reason of the low epilimnetic densities is the grazing by *Ochromonas*, a mixotrophic particle feeder in these lakes (see Chapter 6). Growth of *Chlamydomonas* sp. was determined

to be limited by IC and light (Chapter 3). A single determination of the sestonic epilimnetic in situ C:P ratio in Lake 111 produced high values of 200:1 (w/w) (N. Kamjunke, pers. comm.). The enrichment experiments of Beulker et al. (2002) with 50 µg P L^{-1}, resulted in a three fold increase of the electron transport rate indicating enhanced primary production rates. However, they achieved a measurable increase in photosynthetic rate only by a combined supplementation of IC (1 mg L^{-1}) and P, and suggested temporary coincidence of IC- and P limitation in Lake 111.

Since a potential P limitation of phytoplankton in the acidic mining lakes has been neither proved nor disproved despite previous efforts, the aim of this part of the study was to test cellular P depletion of acidic mining lake phytoplankton in the laboratory and in situ. Most algae rely on the uptake of inorganic P (P_i) from their environment (Beardall et al. 2001). However, since the predominant part of soluble P - at least in circumneutral lakes - is bound in organic compounds such as phosphoric esters, it is not directly available to the algae and has to be hydrolyzed by extracellular phosphatases, producing an alcohol and P_i. Alkaline phosphatase (AP) is located in the cell membrane of many species (species-specific) and expressed in response to P depletion (González-Gil et al. 1998). The ELF-method (enzyme labeled fluorescence) enables to detect AP activity (APA) in single cells in situ by building an insoluble high-fluorescent precipitation at the site of APA (González-Gil et al. 1998). In previous APA bioassays, the detection was performed on bulk natural water using soluble colorimetric or fluorogenic substrates which do not differentiate between different algal taxa or between dissolved and cell-bound AP (Rengefors et al. 2001). With ELF, the fluorescent reaction of distinct cells can be investigated under the microscope and in combination with flow cytometry.

The minimal cellular P quota (Q_0) provides information about the P concentration required for maintenance of the cells. Therefore, I conducted a laboratory experiment where

Chlamydomonas sp. was grown at different growth rates at the transition between light and IC limitation (see Chapter 3) under a fixed P concentration to investigate the cell quota (Q_p = P concentration per cell). Additionally, APA in phytoplankton from lake water samples from different depths was measured with ELF and first tests to separate stained cells by flow cytometry were conducted.

METHODS

Cultures. The influence of the P concentration in the medium on the cellular P content was investigated in *Chlamydomonas* sp. cultures in semicontinuous laboratory experiments. Dilution rates (D) were chosen based on previous experiences (Chapter 3), investigating growth rates around 0.6 d^{-1}. This was achieved by constant daily medium changes. All treatments were performed in twofold with a starting cell density of 5 x 10^4 cells ml^{-1} in all vessels. The medium contained 50 µg P L^{-1} (=1.6 µmol L^{-1}). Light supply was kept at 220 µmol photons m^{-2} s^{-1} with a light/dark period of 16/8 h. CO_2 was supplemented to the atmosphere above the cultures to avoid IC limitation. Cell numbers were determined daily with a cell counter (CASY 1, Model TT, GAT). After the cell densities had reached equilibrium, P content in the cells was measured.

Analysis of P. Total P content of the cell suspension was determined after degradation with $K_2S_2O_8$ and 0.5 M H_2SO_4 at 100°C for 1h. The concentration of soluble reactive phosphorus (SRP) was assessed after filtration of the sample through an acid soaked glass fiber filter. Total and soluble P measurements were subsequently performed by a reduction of the molybdate-complex with ascorbic acid and measured photometrically according to DIN 38 405-D 11-4 and DIN 38 450-D 11-1, respectively.

Calculation of cell quota (Q_P). The cellular P concentration (Q_P) was calculated by subtracting the SRP fraction from the total amount of P and division through cell counts, as

cell volumes did not deviate in the different treatments. The relation of growth rates to Q_p was derived by Droop's equation:

$$\mu = \mu'_{max} (1 - Q_0/Q_P)$$

in which Q_0 is the minimum cell quota (Q_P at μ=0) and μ'_{max} is the apparent maximal growth rate that would occur if Q_P became infinite. μ'_{max} and Q_0 were estimated from the data by non-linear regression curves (SPSS).

Analysis of APA. 1 L of lake water (Lake 111, see Chapter 3) was sampled from 0-2 m (epilimnion) and 6.5 m depth (deep chlorophyll maximum: DCM) on 29 April, 07 and 29 May 2002. The Activity of alkaline phosphatase (APA) was determined following the protocol of the *ELF®97 Endogenous Phosphatase Detection Kit* (*E-6601*, Molecular Probes). The kit consists of three components: A (substrate), B (detection buffer) and C (mounting buffer). The water samples were divided into 4 equal aliquots and centrifuged on the day of sampling for 6 min at 3000 x *g*. Two aliquots were fixed with 1:100 fixative (fixative: 0.01% paraformaldehyde and 0.1% glutaraldehyde) and kept in the dark at 4°C for later analysis in the flow cytometer. One aliquot was used as inoculum for a culture with medium M111 (with saturated P, 50 µmol L^{-1}) as a negative control after 1 week. The last aliquot was divided into four microfuge vials and centrifuged again for 5 min. The supernatant was discarded. Component B (detection buffer) of the ELF-kit was diluted 1:5 with distilled water. Component A (substrate) was diluted 1:20 in the diluted Component B. This substrate solution was filtered through a 0.2 µm pore size filter just before applying to the cell sample. After adding to the pellets of two subsamples (remaining two subsamples: controls, only Component B 1:5), the samples were incubated in the dark at room temperature for 30 min. The reaction was stopped by addition of wash buffer (100 mM TRIS buffer and 5 mM levamisol, pH 8.0). The samples were centrifuged for 5 min and washed for 10-15 min with gentle agitation with wash buffer. This procedure was repeated 3 times. After the last washing

step, as much wash buffer as possible was removed without drying the sample and the pellet was mounted with Component C (mounting medium). The ELF staining was visualized through a standard DAPI longpass filter set with UV excitation (ca. 345 nm) and transmission above 400 nm. The bright yellow-green signal of the stain was easily distinguished against the red autofluorescence of chlorophyll.

Flow Cytometry. Discrimination of ELF-stained cells by Flow Cytometry was carried out once (samples of 29 April) at the Institute of Plant Genetics and Crop Plant Research in Gatersleben, using a FACStar Plus FCM and Cell Sorter (Becton Dickinson, San Jose, CA) equipped with a sort enhancement module (SEM) and an argon ion laser (INNOVA 90-5 Coherent, Palo Alto, CA) exciting UV light with 200 mW. Throughout Germany, few institutions possess a flow cytometer with the necessary filter set and time constraints permitted measurements to be made only once. Chlorophyll *a* (Chl) fluorescence was measured using a 645 nm, long pass filter and ELF fluorescence using a 535 nm, 10 nm band pass filter, controlled by a MacIntosh computer with CellQuest software. FACSFlow sheath fluid (Becton Dickinson) was pressed with 9 psi through a 100-µm nozzle. 10,000 cells were counted to avoid statistical mistakes. Sorting was performed at a nozzle frequency of 15,000 s^{-1}. Prior to measurements, the samples were diluted 1:100 in TRIS-buffer.

RESULTS

Cell quota. The cell densities in the different cultures leveled off according to dilution after a few days, but cultures exhibited increasing cell density fluctuations, especially in the low growth rates (Fig. 5-1). Cell volumes did not change with dilution rate. Cellular P quota (Q_p) related to the growth rate of the culture (Fig. 5-2) as described by the Droop model. This approximation resulted in an estimation of Q_0 of 0.32 pg P $cell^{-1}$ and μ'_{max} of 1.07 d^{-1}.

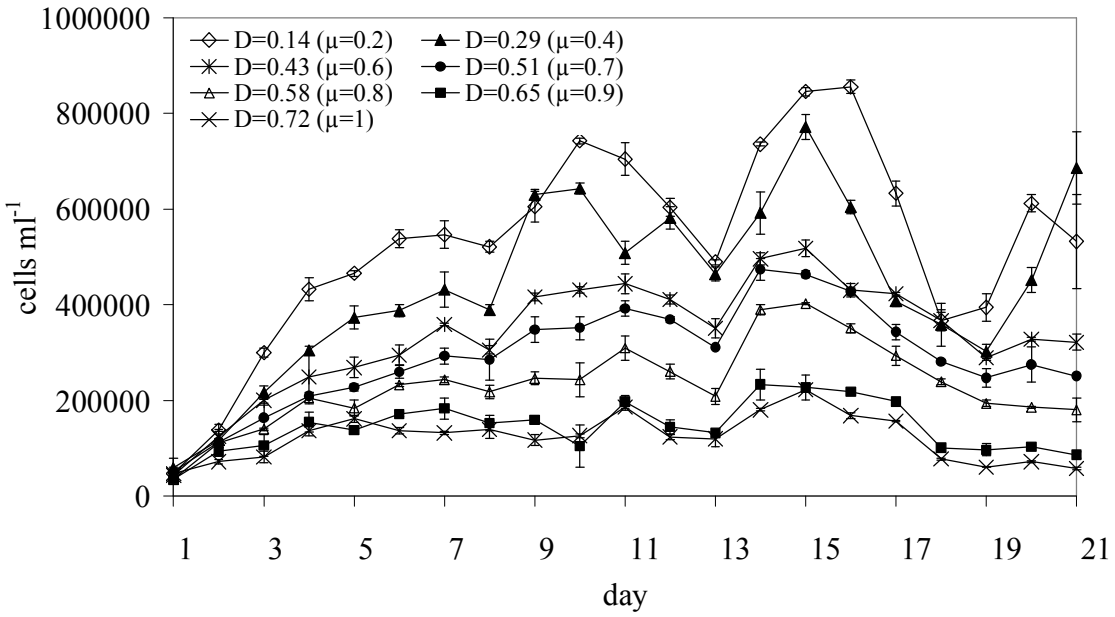


Fig. 5-1. Cell density in the cultures over the time of the experiment. At day 21, P was determined. D=dilution rate, μ=implied growth rate

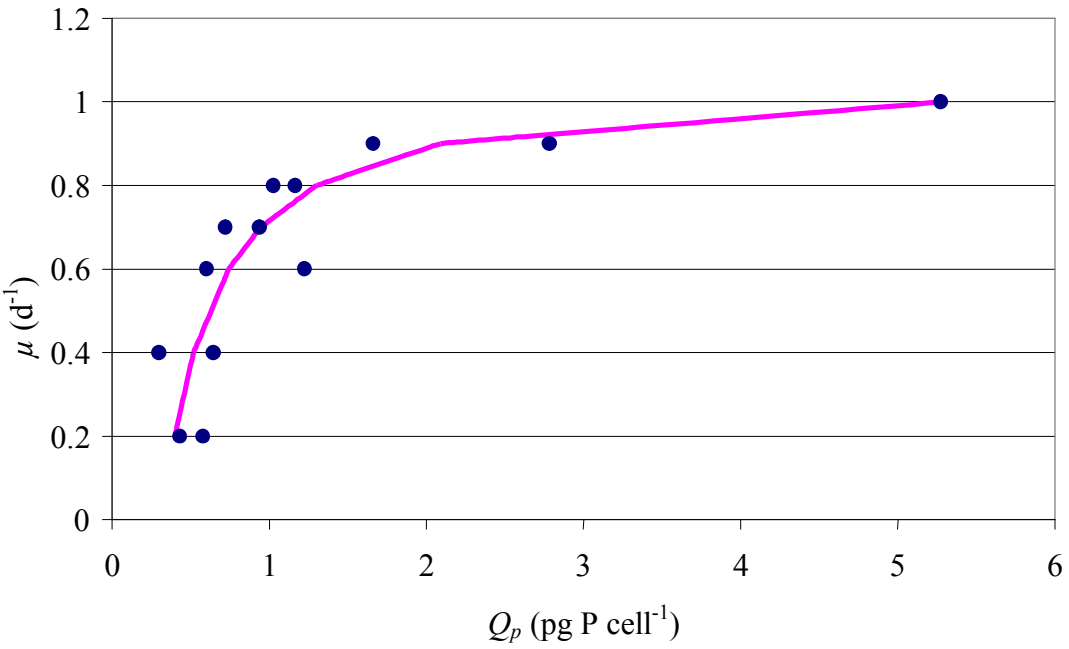
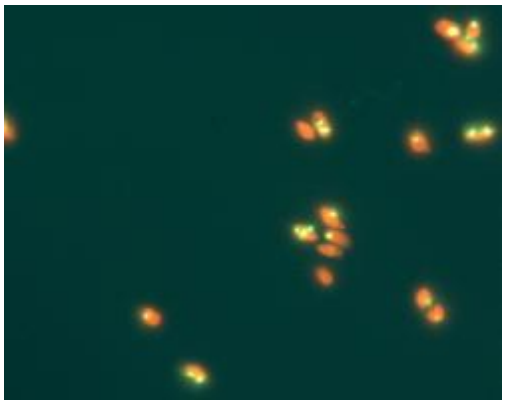


Fig. 5-2. Internal P concentration of the cells (cell quota Q_p) in relation to growth rate (d^{-1}). Curve fitted to the Droop model. Values of μ=0.2 and μ=0.4 may vary according to fluctuations in Fig. 5-1.

Alkaline Phosphatase Activity (APA). The sensitivity of the cells for the ELF reaction was tested with *Chlamydomonas* sp. cultures from a laboratory strain (11A2, compare chapter 2), washed and incubated in filter sterilized lake water for one week. The signal was clearly expressed (Picture 1), meaning that APA was also induced under acidic conditions and the extreme ion concentrations. In the normal P-rich culture (medium M111, see chapter 2), only autofluorescence was detected.



Picture 5-1. *Chlamydomonas* sp. cells stained with ELF. The bright green spots at the site of APA can be clearly distinguished from red autofluorescence. Scale: 0.5 cm ≈ 10 µm.

The ELF signal was detected in all in situ samples but varied in intensity (Table 5-1). A low signal in most of the observed cells had to be discriminated against a high signal in only a small fraction of the cells. On 29 April and 7 May, the signals were low in most cells. 29 April and 29 May 2002 there were obvious differences between samples from the epilimnion and from the DCM. The brightest signal in most cells was expressed in the sample from the DCM on 29 May.

A comparison of the reaction of *Chlamydomonas* sp. and *Ochromonas* sp. to the ELF staining was hampered by methodological problems. The buffers of the ELF-kit have a high ionic concentration which caused the cell-wall-free *Ochromonas* cells to burst. Indeed, no intact

Ochromonas cells were found in any ELF-stained field sample, although they were present in the non-stained water samples.

Table 5-1. Expression of the ELF signal in Lake 111 water samples during spring. 0-2 m: epilimnion, 6.5 m: DCM. +: overall low response in the sample, ++: medium response, +++: high response.

Date	Sampling depth (m)	ELF signal	
29 Apr 02	0-2	+	Few cells with high signal
	6.5	++	Most cells with low signal
07 May 02	0-2	+	Few cells with low signal
	6.5	+	Few cells with low signal
29 May 02	0-2	+	Few cells with low signal
	6.5	+++	Most cells with high signal

Flow Cytometry. The detection of ELF response by flow cytometry could only be conducted once with samples from 29 April to test the applicability of the method. The results of discrimination by Chl-fluorescence and ELF-fluorescence for the two depths against the control without ELF and the control with saturated P are shown in Fig. 5-3. All the graphs reflect one cloud of fluorescing cells in the left upper corner (red dots) which can be attributed to Chl-fluorescence. In the ELF treated water samples (middle graphs in Fig. 5-3), there was a shift of these cells to the right, due to higher response of the fluorescence contributed to ELF (green dots). This shift was not detected when cells were incubated with P-saturated medium and subsequently treated with ELF (right graphs in Fig. 5-3). Note that Chl fluorescence decreased in these cases. The outlying particles in the diagonal line of some graphs originate from bacteria and detritus (low/no Chl fluorescence; black dots).

After sorting the cells from the autofluorescent and ELF-stained accumulations by the different expression of fluorescence at 530 nm, the two suspensions were checked under the microscope. Clear proof was obtained for the stated discrimination between ELF-stained and unstained cells, as shown in Fig. 5-3.

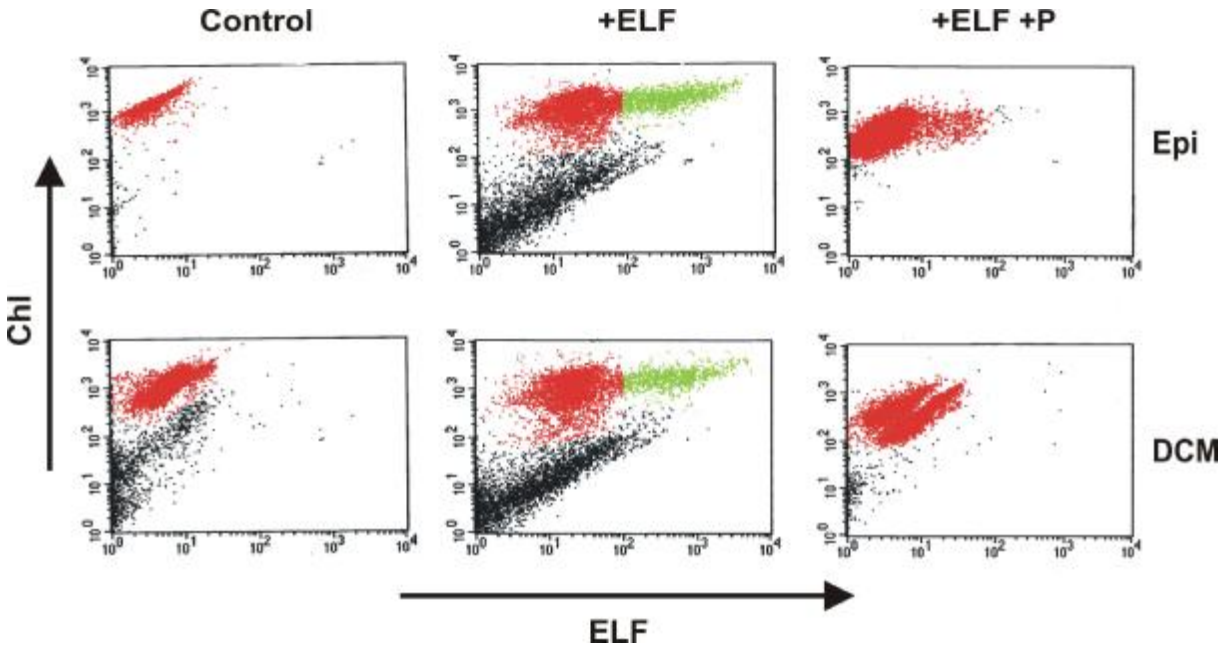


Fig. 5-3. Plots of flow cytometry detection of Lake 111 samples from 29 April 2002. Upper graphs: Epi=epilimnetic water samples (0-2 m), lower graphs: water sample from the DCM (6.5 m). Left graphs: Control (without ELF substrate), middle graphs: with ELF substrate, right graphs: Field samples after incubation with P-saturated medium and ELF substrate. Red dots: autofluorescence (Chl), green dots: ELF staining, black dots: bacteria and detritus. x-axis: ELF fluorescence (emission 530 nm), y-axis: chlorophyll a fluorescence (emission 680 nm), relative fluorescence units in (LOG) scales.

DISCUSSION

In this study, I found a clear relationship between the cellular P quota (Q_p) and growth rate in *Chlamydomonas* sp. from Lake 111. The minimal cell quota (Q_0) represent the amount of P associated with the structural and metabolic components that are essential for cellular integrity and viability (e.g. cell walls and membranes, DNA activity responsible for

maintenance metabolism). The Q_0 in the *Chlamydomonas* sp. from the acidic lake described in our study was much higher (approximately 10 fmol/cell) than that of comparably sized *Chlamydomonas* sp. (3.5 fmol/cell, Grover 1989) and *C. geitleri* (2.2 fmol/cell, Vyhnálek 1990) cells from circumneutral conditions. These numbers indicate that *Chlamydomonas* sp. from the acidic mining lake requires a higher concentration of P for cell maintenance. This suggests that at a given SRP concentration, maximum phytoplankton standing stocks in the acidic lakes are lower than in neutral lakes (Sas 1989).

The data presented here do not describe a relationship between growth and P_i concentrations in the medium (Monod relation), because very slow growing algal populations reduce the P_i concentrations to very low levels that can only be determined with radio-active measurements. A competitive ability comparison with other algal species based on parameters from this 'Monod' relation (μ_{max}/K_s and R^*, Grover 1989) was therefore not possible. Adaptations in long-time (20 min) uptake kinetics have resulted in a 10 fold increase in affinity (E. Spijkerman, pers. comm.). Both, maximum uptake rate and the affinity constant for uptake were higher in the P-limited than in the P-replete culture. A dependence of the uptake kinetic on the internal P pool is therefore likely to occur in *Chlamydomonas* sp.

Applying the expected in situ growth rates in the epilimnion of Lake 111 (ca. 0.6 d^{-1}, Chapter 3), the cell quota Q_p can be calculated from Fig. 5-3 to ca. 0.75 pg P $cell^{-1}$. Using this Q_p value and presuming that the measured SRP of 7 µg L^{-1} is fully available, *Chlamydomonas* sp. can potentially realize a maximum cell density of ca. 1 x 10^7 cells L^{-1}. The realized densities for *Chlamydomonas* in Lake 111 are approximately one order of magnitude lower in the epilimnion (1 x 10^6 L^{-1}, Table 3-2). Potential reasons might be i) top-down control (investigated in Chapter 6) ii) an incomplete availability of the SRP (not investigated here) or iii) an increased P demand under CO_2 limitation. The Q_p data generated in this study were measured in cultures with a non-limiting supply of CO_2, but the de facto realized Q_p in situ

would in reality be expected to differ due to different P uptake kinetics at lower CO_2 concentrations. At present I am unaware of any algal literature comparing the influence of P limiting conditions while co-inducing an IC limitation. The IC limiting conditions have possibly led to the development of a carbon concentrating mechanism in these algal cells which may account for the increased P requirements per algal cell observed, since a CCM requires enhanced ATP levels (Kaplan and Reinhold 1999). Hence, Q_0 values under IC limitation might be higher than the values measured under non-limiting CO_2 concentration (Fig. 5-2), resulting in lower potential cell densities.

The expression of the ELF signal allowed the microscope discrimination of the amount of cells with detectable signal and the intensity of the signal in individual cells. The fixative used did not change the results of the ELF signal and seemed to be useful for a longer storage of the cells. During the period investigated, *Chlamydomonas* cells were stained by ELF in all samples. At the DCM, the overall response was higher than in the epilimnetic samples on 29 April and on 29 May 2002, in particular (Table 5-1, third column). Since the SRP concentrations were high enough throughout the water column (7 µg L^{-1}), competition between the cells in the DCM cannot explain the high ELF signal expressed. Potentially a major part of the SRP is not bioavailable for the algae at this depth. However, a further possibility exists; top-down control by grazers. Grazer densities (*Ochromonas* sp.) suggest that the grazing pressure on *Chlamydomonas* sp. at the DCM is lower than in the upper strata (Chapter 6). This agrees with the much (ca. 15 times) higher biomass in the DCM and a stronger P depletion (APA) at this depth. Rengefors et al. (2001) conducted the first investigation of freshwater phytoplankton applying the ELF-method in situ in Lake Erken (Sweden). They observed the highest proportion of ELF-labeled cells at the end of May and beginning of June. A seasonal comparison with our field observations will be subject of future investigations and was not possible within the present study. The results so far suggest that

Chlamydomonas sp. expresses APA in response to low available P_i. High phosphatase activity may be a general characteristic of acidic lakes due to the formation of aluminum-organic P complexes (Joseph et al. 1995). Thus, even though the SRP concentrations in the lake are sufficiently high to enable P-unlimited growth in neutral lakes, they may nevertheless be insufficient for growth in the ion-rich mining lakes, since a large fraction of the SRP may be complexed and unavailable for uptake by the phytoplankton.

In contrast to the remarkable occurrence of the ELF-signal in *Chlamydomonas* cells, the second dominant phytoplankter *Ochromonas* did not survive the staining process due to sensitivity of its cell wall free membranes. P limitation is, however, considered unlikely in *Ochromonas* since this alga is a particle-grazing organism (in contrast to the osmotrophic *Chlamydomonas*) (Sanders et al. 2001); *Ochromonas* obtains much of its P requirements by feeding on bacteria. From data generated by Hochstädter (2000) one can easily calculate that feeding on bacteria is an efficient strategy to avoid P limitation.

The different expression of the ELF-signal in cells of one population reflects the individual conditions of microenvironments and individual internal P status each cell experiences (Rengefors et al. 2001). Thus, the ELF technique provides valuable additional insight into the P status of individual cells, while the exclusive interpretation of C:P ratios gives only information on the population level.

The discrimination of ELF-stained cells by means of flow cytometry was shown to be a useful tool. The results clearly exhibited the difference between autofluorescence and ELF-fluorescence. ELF-stained algae could be separated from bacteria and detritus. This method should be used in further analyses of water samples to investigate seasonal changes. However, the investigation of field samples can cause some problems as very low cell densities result in a prolonged counting, and low density populations may be underestimated in mixed samples. Thus, the microscopic determination should be applied in parallel.

The aim of this study was to test the induction of APA in *Chlamydomonas* sp. and to help to untangle the conflicting results concerning P depletion of phytoplankton in the acidic lakes. It was possible to detect the induction of APA in single *Chlamydomonas* cells from field samples, and to demonstrate its dependence on their previous P supply (Fig. 5-3). This method still needs some adaptations to more sensitive phytoplankton species, but it can be recommended as useful tool for further investigations in these environments. According to expectations, the cell quota Q_p strongly depended on growth rates and Q_0 was higher than under circumneutral conditions.

ACKNOWLEDGEMENTS

I like to thank Elly Spijkerman for the support with designing the experiments, establishing the contact with Marco Dignum from Amsterdam University, and revising the manuscript. Marco gave helpful advice about the handling of the ELF-staining. Armin Meister, Institute of Plant Genetics and Crop Plant Research Gatersleben, assisted with the use of the institute's flow cytometer with the necessary filter combination. He explained every detail about the instrument and devoted a whole day of his time to help with the measurements. Tobias Lange assisted with the semicontinuous culturing and Cornelia Hesse measured the Chl. Ursula Gaedke and Jörg Tittel gave critical and valuable comments on the manuscript.

6 DEEP CHLOROPHYLL MAXIMA OR SURFACE CHLOROPHYLL MINIMA?

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Submitted to **Proceedings of the National Academy of Science (PNAS)**

ABSTRACT

The vertical abundance pattern of algae in nutrient poor ocean areas and in many freshwater lakes is typically characterized by cell concentrations that are low at the surface but increase with depth, often resulting in a pronounced deep chlorophyll maximum (DCM). From their location at strata boundaries, it has been concluded that resource availability controls DCM formation. However, this contrasts with observations indicating equal or higher cellular growth of algae at the surface and suggests instead the influence of loss factors. Here, we

demonstrate that in a low-diversity plankton system in an acidic lake, the vertical gradients of food algae and bacteria were the result of a steep surface reduction of these organisms by mixotrophic grazers. Mixotrophs are able to use different food and energy sources. By combining light and particulate food, mixotrophs out-competed specialist grazers in illuminated surface strata. They steeply reduced the abundance of their prey, generating a surface chlorophyll minimum, rather than the apparent DCM. We propose that this mechanism controls the formation of DCM in many aquatic environments. In contrast to common ecological assumptions, the data indicate that the low efficiency use of different resources can be a superior strategy to specialization, even under stable environmental conditions.

INTRODUCTION

Deep chlorophyll maxima (DCM) represent accumulations of phototrophs (cyanobacteria or eukaryotic algae) in sub-surface strata and occur to depths of approximately 120 m. DCM are one of the most striking characteristics of nutrient poor waters, such as central ocean gyres and clear water lakes (Cullen 1982, Abbott et al. 1984). Although cell specific chlorophyll contents have been observed to increase with depth, DCM typically represent biomass maxima and often constitute a substantial proportion of the phototrophic biomass (Coon et al. 1987, Estrada et al. 1993, Agawin and Agusti 1997, Gross et al. 1997, Brock et al. 1998, Arin et al. 1999). The formation of DCM has traditionally been interpreted from a bottom-up perspective by enhanced nutrient availability at depth (Carney et al. 1988, Letelier et al. 1993, Reynolds 1997, Gin et al. 1998, Klausmeier and Litchman 2001). The biomass achievable at a given nutrient concentration, however, depends on cellular growth. Higher growth rates, e.g. of algae to compensate for grazing losses, require higher external and internal nutrient concentrations and are therefore associated with lower achievable algal biomass. This means, that even in situations when growth balances losses, nutrients alone cannot sufficiently explain biomass patterns. While light or mineral nutrients may limit instantaneous cellular growth, only nutrients like phosphorus can limit biomass as they are not supplied recurrently.

Several studies demonstrated high cell division rates at the DCM (Partensky et al. 1996, Agawin and Agusti 1997, Gross et al. 1997). Such sustained growth at the DCM implies that phototroph abundance is the result of both production *and* losses. Grazing is known to be the most important loss process for these phototrophs, facilitating sustained growth via nutrient remineralization (Brock et al. 1998). If grazing, rather than resources, stimulates the formation of the DCM, vertical gradients in grazing pressure must balance growth over a period of many months, a situation which may arise via the following mechanism:

According to the mechanistic resource competition theory (Tilman 1982, Rothhaupt 1996b), in the dark specialist grazers (phagotrophs) reach zero net population growth at food concentrations (F_0) lower than mixotrophic grazers ($F_{0,P}<F_{0,M}$). Pigmented mixotrophic grazers are able to use organic carbon (e.g. prey items) and light as substitutable energy sources. Thus, conversely, in the light mixotrophs require lower food densities than the non-pigmented phagotrophs ($F^L_{0,M}<F_{0,P}$) and are able to out-compete them (Rothhaupt 1996b) (Fig. 6-1). We hypothesize that high phototroph abundance in the DCM reflects higher phagotroph food thresholds ($F_{0,P}$) compared with those of mixotrophs illuminated at the surface ($F^L_{0,M}$). This may not apply to some lakes where the DCM is formed by phototrophs less susceptible to grazing (e.g. colonial chrysophytes (Fee 1976)) and to systems in which an obligate resource for the phototrophs is only available within a deep boundary layer (e.g. reduced sulfur for pigmented sulfur bacteria). Although mixotrophy includes many forms, in the model and in our discussion, we define our use of the term ”mixotroph” to the combination of phagotrophy and phototrophy. This type of mixotrophy must have been evaluated functionally by their possession of pigments and their experimentally demonstrated ability to take up prey (Arenovski et al. 1995; Supporting information, section A).

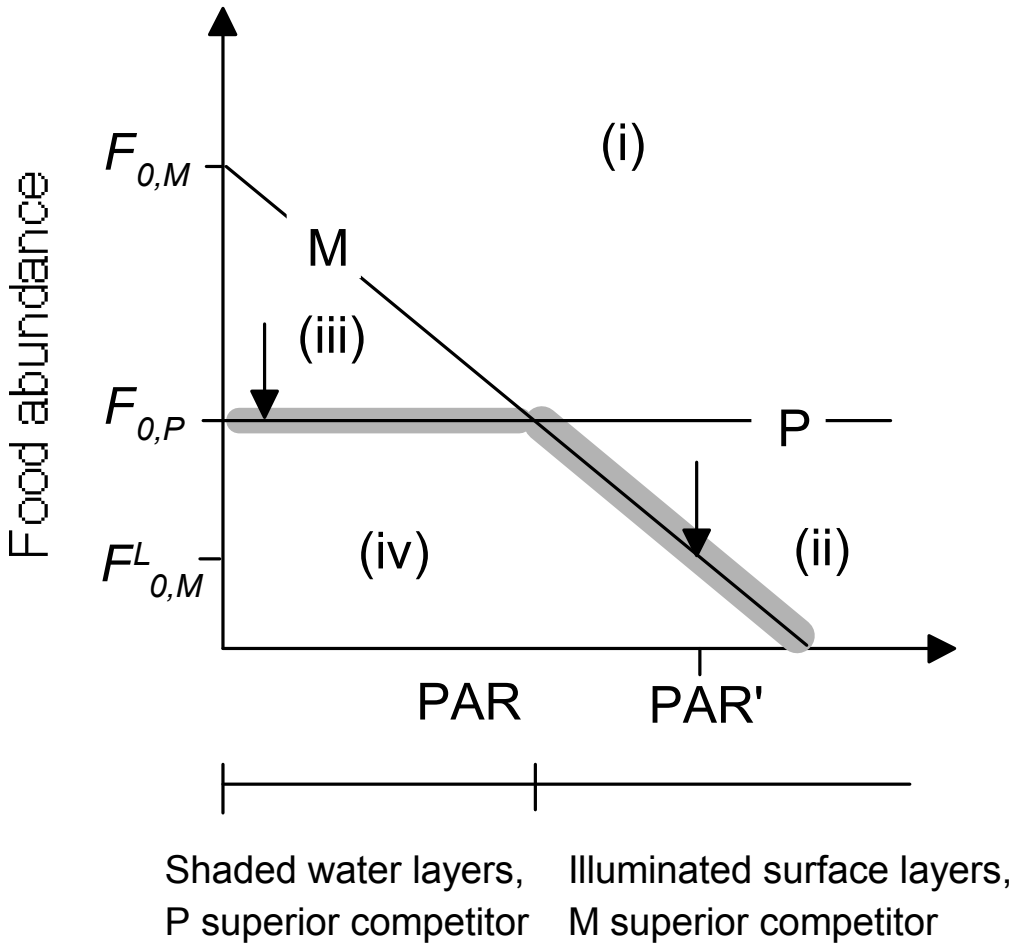

Fig. 6-1. Application of the mechanistic resource competition theory (Tilman 1982, Rothhaupt 1996b) to explain the mechanisms of DCM formation. Lines indicate resource combinations at which the net population growth of a phagotrophic (P) and a mixotrophic (M) organism are zero, the zero net growth isoclines (ZNGI). Sections i-iv show resource combinations allowing the growth of M+P (i), M (ii), P (iii) and neither M nor P (iv, only theoretically). $F_{0,P}$ and $F_{0,M}$ refer to the zero net growth food concentrations in the dark. The consumption vectors (arrow) indicate resource changes by grazing assuming no variation of PAR attenuation due to varying predator or prey densities. Starting in section (i) grazing by M or P reduces the food abundance until the population growth of the superior competitor equals zero (thick grey line), indicated by the lower F_0 derived from the ZNGI and a given PAR. In shaded strata (DCM), $F_{0,P}$ determines the food abundance. $F^L_{0,M}$ represents phototroph abundance at the surface as a function of PAR at half epilimnion depth (PAR') assuming vertical mixing.

METHODS

We tested our hypothesis on the low diversity planktonic food web of an acidic mining lake (Lake 111, Germany, pH 2.6-3.3 (Friese et al. 1998, Wollmann et al. 2000)) with a distinct DCM (Fig. 6-2). Physical data including *in situ* fluorescence were obtained using an automatic probe (Idronaut, Brugherio, Italy). Photosynthetic available radiation (PAR) was measured using spherical quantum sensors (SPQA, Li-Cor). We took water samples using tubing mounted near the probe's depth sensor. Samples were pre-acidified with H_2SO_4 to a final concentration of 0.3 % and then fixed with Lugol's Iodine to prevent iodine precipitation under the lake water conditions.

Protozoan cell density was determined microscopically by counting in sedimentation chambers. Bacterial numbers were quantified by means of epifluorescence microscopy after staining with acridine-orange. Prior to staining, the Lugol's color was removed by adding a few drops of 0.1 N sodium thiosulfate. Biovolumes were calculated by approximation to simple geometrical forms.

Experiments were performed in the laboratory. We conducted grazing experiments to test the grazing impact of the mixotroph, *Ochromonas*, on the *Chlamydomonas* food algae and competition experiments of mixotrophs and phagotrophs, the ciliates *Oxytricha,* for prey. Bacteria (strain 99P5 University of Potsdam), the flagellates *Chlamydomonas* (11A2) and *Ochromonas* (1B3) and ciliates *Oxytricha* (99X4) were isolated from field samples. Each isolate was pre-cultured at 20 ± 1 °C in an incubator simulating the specific *in situ* light spectrum (Koschorreck and Tittel 2002). A PAR of 150 µmol photons m^{-2} s^{-1} was supplied continuously. We measured the PAR inside the culturing flasks with a quantum sensor (QSL-101, Biospherical). We used an inorganic nutrient replete medium, simulating the lake's chemical conditions (Bissinger et al. 2000). The experiments were run in duplicates. Abundance was monitored by microscopical cell enumeration.

Further laboratory experiments were conducted to test the growth of food algae and bacteria under *in situ* conditions and ambient dissolved organic carbon (DOC) quality. We collected water from Lake 111 on 7/24 (2.5 m depth), 8/22 (2.5 m), 8/30 (3.6 m), 10/10 (6.0 m) and

11/14 (8.5 m) 2000. The water was filtered with low pressure (10 kPa) using capsules (0.22 µm pore size, MPGL 06 GH2, Millipore). We employed bacterioplankton collected *in situ* that was adapted to the ambient DOC. Bacteria were taken from the sampled water and separated by gravity filtration through a 0.8 µm filter. The natural bacterioplankton and pre-incubated *Chlamydomonas* were inoculated in separate cultures at low densities (<2% of lake abundances) and maintained under a 16:8 hours light dark cycle. Due to the low pH, inorganic carbon is only present in the form of dissolved CO_2. We therefore adjusted the medium's CO_2 content to *in situ* conditions by pumping air containing 5 % CO_2 into the incubation chamber. The growth rates over the logarithmic growth phase (6-30 days) were calculated for pooled data from two replicates.

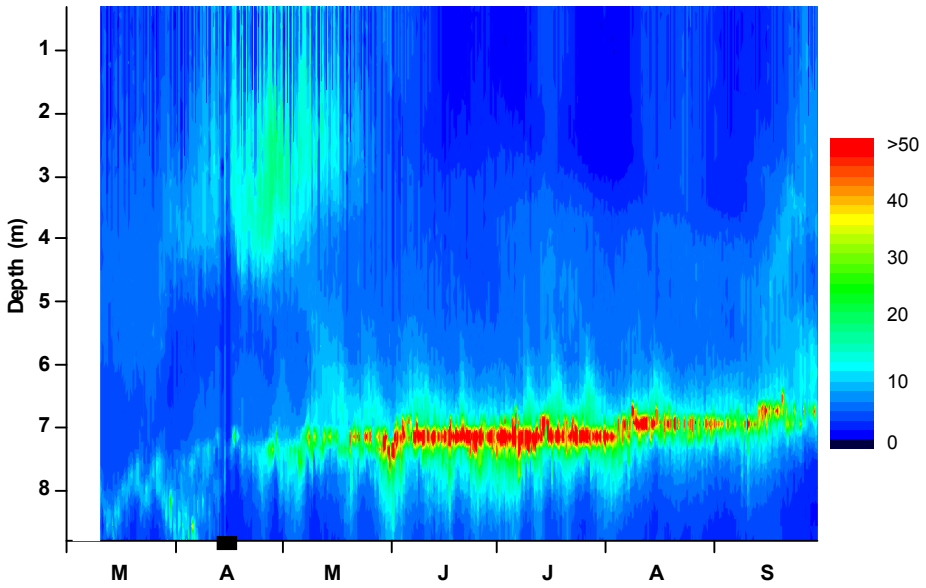

Fig. 6-2. The 1999 deep chlorophyll maximum in Lake 111. Relative units of *in situ* fluorescence (rough indicator of chlorophyll concentration) are shown. The highest summer fluorescence values corresponded to a chlorophyll *a* concentration of approximately 60 µg L^{-1} (29 June, 7.0 m depth, linear scale). No data from 13. to 17. April (horizontal bar).

Table 6-1. Growth of prey organisms at DCM- and surface conditions.

Growth rates of separate cultures of natural bacterioplankton (Kamjunke et al., submitted) and phototrophs (*Chlamydomonas*) (μ ± SE). We used sterile filtered Lake 111 water from different depths. Applied PAR corresponded to that observed *in situ* at depth, assuming a surface PAR of 630 µmol photons m^{-2} s^{-1} (cloudy summer day). To prove unequivocally that available nutrients allowed phototrophs growth in the DCM (6 m), we doubled PAR to 7 µmol photons m^{-2} s^{-1} in this treatment.

depth (m)	DOC (µmolC L^{-1})	PAR (µE $m^{-2}s^{-1}$)	temperature (°C)	μ Chlamyd. (d^{-1})	μ bacteria (d^{-1})
2.5	108	60	17.0	0.60 ±0.06	1.05 ±0.05
2.5	67	60	22.1	0.60 ±0.03	1.01 ±0.06
3.6	75	20	16.4	-0.02 ±0.03	0.34 ±0.06
6.0	308	7	10.3	0.05 ±0.01	0.15 ±0.01
8.5	242	2	7.8	-0.01 ±0.01	0.13 ±0.02

RESULTS AND DISCUSSION

Important functional groups in acidic Lake 111 were represented by only one or a few species in the food web (Wollmann et al. 2000). These comprised the DCM forming phototroph, *Chlamydomonas* sp.; the pigmented mixotrophic grazer, *Ochromonas* sp.; phagotrophic protozoans (heliozoans and ciliates); and non-pigmented bacteria. The mixotrophs were the dominant grazers throughout the water column, particularly in illuminated surface strata (Fig. 6-3). Due to their low specific chlorophyll *a* content (1.7 and 20.2 µg mm^{-3} in light saturated *Ochromonas* and *Chlamydomonas* cultures, respectively), the mixotrophs did not enhance surface chlorophyll concentrations significantly. Phagotroph biomass was low. Mixo- and phagotroph prey biomass (phototrophs and non-filamentous bacteria) was significantly lower in illuminated than in shaded strata. These observations agreed well with the mechanistic theory and support our hypothesis that grazing by mixotrophs steeply reduces phototrophic biomass at the surface. To exclude alternative hypotheses we needed to show that (i) cellular growth of prey organisms (but not necessarily population growth) occurred at and above the

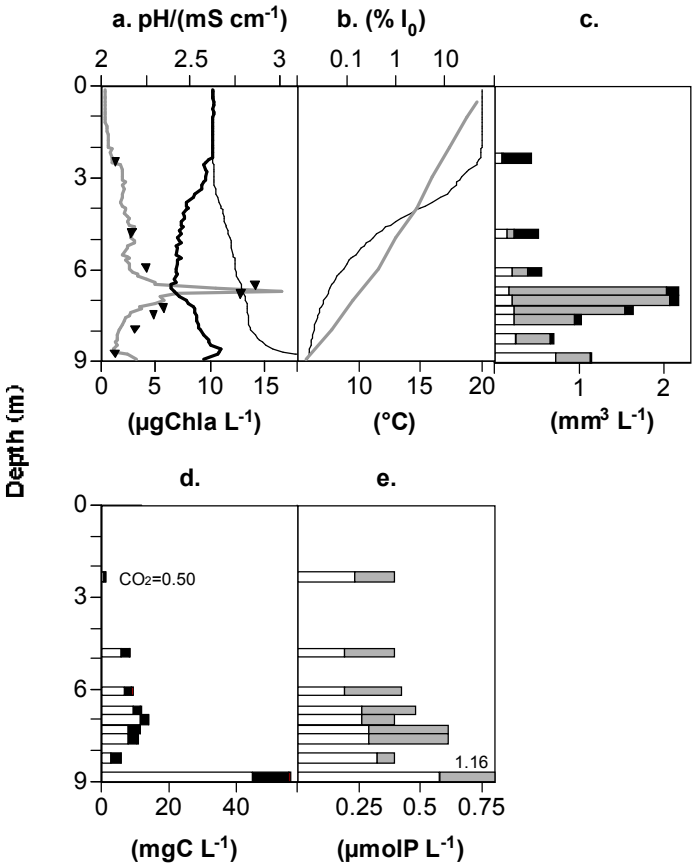

Fig. 6-3. Vertical distribution of plankton taxa, physical parameters and nutrients in Lake 111 (15 Sept. 1999). (*a*) Chlorophyll *a* (Chl *a*, triangles) and *in situ* fluorescence (gray line), pH (thin line) and conductivity (χ_{25}, thick line). Conductivity indicates the presence of a mixed epilimnion (0-3 m), an intermediate layer (down to 6.5 m) and a deeper water layer. (*b*) Temperature (black), PAR (gray). (*c*) Biovolume of non-filamentous bacteria (white), phototrophs (*Chlamydomonas*, gray) and mixotrophs (*Ochromonas*, black). Median biovolumes of non-filamentous (edible) bacteria from several sampling occasions were lower in epilimnion than in layers down to the DCM (3.6-7.6 m depth) (11 vs. 17 samples, p=0.0005, two-tailed *U*-test). Phototrophs were also higher in the depth (p=0.0024). Mixotrophs exhibited higher biovolumes in epilimnion (p=0.0147). Phagotrophs (heliozoans plus ciliates) and filamentous bacteria (less edible) did not vary in the vertical range (not shown, p=0.2561 and 0.0509, respectively). Phagotroph biovolumes were 1.6 orders of magnitude lower than those of mixotrophs. Non-protozoan phagotrophs were of minor importance (Wollmann et al. 2000). (*d*) CO_2 (white) and DOC (black). (*e*) Total phosphorus (whole column) and soluble reactive phosphorus (SRP, white). Nitrogen was found in excess (≥214 µM NH_4^+). We did not detect H_2S.

DCM, (ii) the mixotrophs consumed the equally sized phototroph, (iii) mixotrophs in the light had a lower F_0 than mixotrophs and phagotrophs in the dark and, therefore, (iv) the mixotrophs out-competed the phagotrophs in the light.

The growth of the phototroph in lake water from different depths (Table 6-1) indicated sustained cellular growth in the DCM and above. Growth rates of phototrophs and bacteria were highest in surface strata. Prey abundances, in contrast, were much higher at depth (Fig. 6-3c), corroborating the hypothesis that loss processes controlled abundances.

The ingestion of phototrophs by mixotrophs was microscopically and experimentally verified: Inoculated at typical epilimnion densities, phototroph net growth was lower when grown together with the mixotrophs than alone (0.49 ± 0.02 and 0.58 ± 0.02 d^{-1}, respectively). It is known that *Ochromonas* feed on algae (Olrik and Nauwerck 1993). The capability to ingest algae significantly larger than its own cell size has already been demonstrated for the closely related mixotrophic flagellate *Poterioochromonas malhamensis* (Zhang and Watanabe 2001).

We determined the food thresholds (F_0) of mixotrophs and phagotrophs experimentally using bacterial prey (Fig. 6-4). We used bacteria rather than phototrophs as prey because bacterial densities indicating F_0 were assumed to be more stable in the dark treatment. In the light, bacteria were grazed to lower densities than in the dark (minimum 0.36 10^8 and 1.10 10^8 L^{-1}, respectively). The final biomass ratio of mixotrophs to phagotrophs was 843:1 in the light and 6:1 in the dark. These results generally conform to the mechanistic competition theory and verify our hypothesis of a steeper prey reduction in the light than in the dark. The predicted competitive advantage of phagotrophs in the dark (Fig. 6-1) was less than expected. In the light, grazing by phagotrophs on mixotrophs may have prevented both further mixotrophic growth and the complete competitive removal of phagotrophs (Fig. 6-4a).

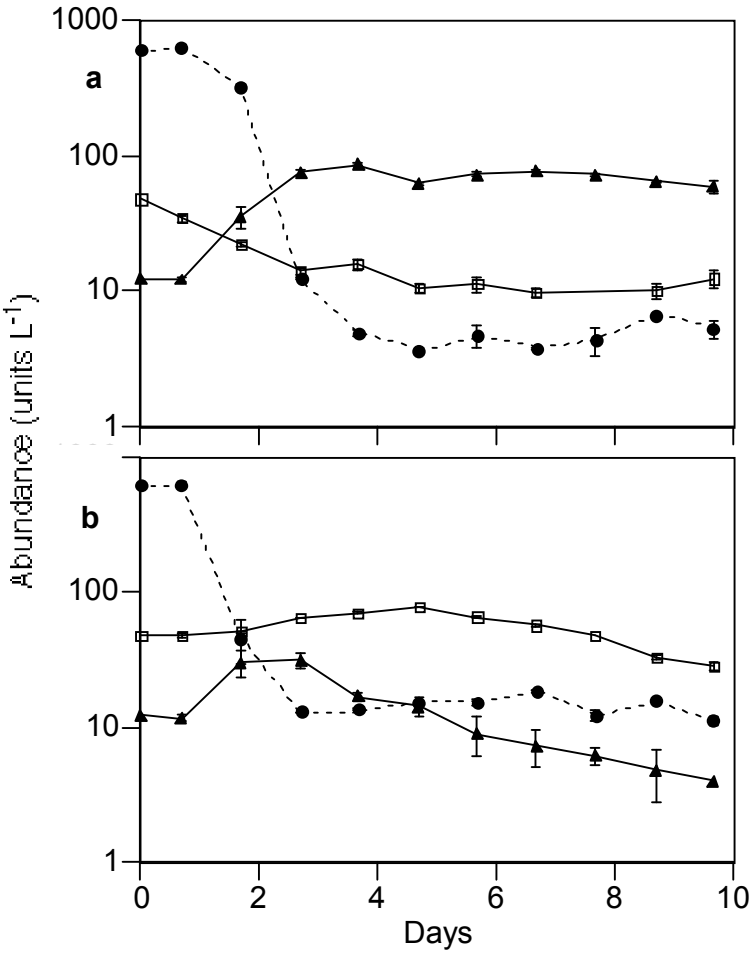

Fig. 6-4. Competition between mixotrophs and phagotrophs for bacteria in the light (**a**, 150 µmol photons $m^{-2}s^{-1}$, 20±1 °C) and in the dark (**b**). Symbols represent the means and ranges of two replicates: circles - 10^7 bacteria; triangles - 10^6 mixotrophs (*Ochromonas* sp.); cubes - 10^2 phagotrophs (the ciliate *Oxytricha* sp.).

The lower limit for the occurrence of phototrophs is set by their energetic requirements which are met by light and potentially by the uptake of DOC. Although *Chlamydomonas* has the potential to use DOC (experimental results (Bissinger et al. 2000; supporting information, section B) and cf. Table 6-1, 3.6 and 6 m depth), we refer to it as phototroph for means of simplicity. Within this energetically feasible depth range, phototroph abundance is determined by the F_0 of the dominant grazer (Fig. 6-3 and 6-4). Because mixotrophs dominated, the abundance of phototrophs should follow their ZNGI (Fig. 6-1) resulting in a pronounced DCM due to exponential light attenuation with depth (Fig. 6-3). Therefore, the observed

formation of the DCM in Lake 111 was indeed the result of a steep reduction of phototroph abundance by the mixotrophs in illuminated strata. Additionally, higher mixotroph abundances at the surface illustrate that the biomass of a consumer depends on prey production and not directly on prey abundance. Although prey abundances are low at the surface, prey production rates inferred from the specific growth rates given in Table 6-1 are higher than in deeper layers.

In biological oceanography literature, we found a number of studies clearly illustrating the ideas presented, although the authors themselves did not link their observations to the mechanistic theory (Agawin and Agusti 1997, Havskum and Riemann 1996). For example, Havskum and Riemann (1996) investigated the role of bacterivorous flagellates in the Bay of Aarhus, Denmark and concluded "... that mixotrophic flagellates constituted half of the pigmented biomass in the nutrient-depleted top layer of the Bay of Aarhus. These mixotrophs were also responsible for almost the entire flagellate grazing on bacteria. The bacteria were grazed down to a low level, $<1 \times 10^6$ ml^{-1}, and our results strongly suggest that no flagellate group was able to sustain its growth solely on bacterivory... In the deeper, nutrient-rich environment, bacterivorous pigmented flagellates accounted, on average, for only 9 % of the pigmented biomass. Here bacterial abundance was higher and colorless flagellates were mainly responsible for the grazing on bacteria."

To demonstrate the effects of combining the use of two resources (i.e. light and organic carbon) in nature, we chose a system with a low diversity food web that allowed us to employ all of the constituent species in our experiments. We acknowledge that the extreme environmental conditions in our system provoke questions about the generality of the results presented here; other more common systems are larger, not acidic, or more diverse. Nevertheless, the basic ecological mechanisms of species interaction, as illustrated in Fig. 6-1, have been proven to be independent of scale, habitat type or the species involved (e.g. resource partitioning (Connell 1961, Hylleberg 1976)).

Mechanistic theory predicts that mixotrophs reduce food abundances steeply in surface waters if (i) significant losses to higher trophic levels do not occur, allowing the mixotrophs to take full advantage of their strategy (Rothhaupt 1996a and b), (ii) organic carbon (prey items) is

available to mixotrophs and (iii) the mixotrophs are able to combine light and organic carbon resource use. The first prerequisite is rather given in oligotrophic areas because predation generally increases with enrichment. In accordance with the second, pigmented flagellates ingesting prey have been evaluated as important planktonic constituents, as they contribute to the consumption of the predominating small phototrophs (Sanders et al. 2000, Havskum and Hansen 1997). In nutrient poor areas, phototrophs generally consist of small cells which are efficiently consumed by protozoans within the microbial food web (Gieskes and Kraay 1986, Sherr et al. 1991, Havskum and Riemann 1996). In accordance with the third, the steep reduction of prey abundances by mixotrophs when light is supplied has been exemplified under laboratory and field conditions (Havskum and Riemann 1996, Rothhaupt 1996a). Moreover, a number of studies have shown that mixotrophs are abundant and active in illuminated surface waters (Nygaard and Tobiesen 1993, Dolan and Marrase 1995, Havskum and Riemann 1996, Pitta and Giannakourou 2000, Pitta et al. 2001). For example, in the oligotrophic Sargasso Sea, up to 50% of nanoplanktonic algae in surface waters ingested bacteria and this proportion decreased with depth, not exceeding 0.5% at the DCM (Arenovski et al. 1995). Indeed, evidence for the significance of loss processes in DCM formation came from studies in marine and limnetic environments showing that cell division rates of phototrophs in surface layers were higher than or equal to those at DCM (Agawin and Agusti 1997, Partensky et al. 1996, Padisak et al. 1997).

The emerging picture makes sense in the context of the mechanistic theory and led us hypothesize that light modifies grazing activity throughout the vertical water column. Further studies are required to determine conclusively whether mixotrophs combining photo- and phagotrophy represent an unexpected force increasing phototroph turnover rates in nutrient poor surface systems. The use of many resources is connected with enhanced basic metabolic requirements and decreasing efficiency using each single resource (Rothhaupt 1996b). Although current ecology paradigm predicts that specialization should be the most successful strategy for survival under stable conditions (MacArthur and Connell 1966, Paine 1979), our data indicate that the use of several resources with lower efficiency can be an equally successful strategy in nature.

SUPPORTING INFORMATION

A. The mixotrophic nature of *Ochromonas* in Lake 111

For growth experiments, three different treatments were chosen: (a) phototrophic growth (PAR 150 µmol photons m^{-2} s^{-1}, very low bacterial densities of $\leq 10^8$ cells L^{-1}, medium free of organic compounds except vitamins (1)), (b) phagotrophic growth (dark, high bacterial densities of ca. 10^{11} cells L^{-1} in medium with glucose supplement of 20 mg C L^{-1} final concentration) and (c) mixotrophic growth (as for phagotrophic growth except PAR supply of 150 µmol photons m^{-2} s^{-1}). It should be noted, that growth in (a) did not represent pure phototrophic growth because few bacteria were still present. However, contribution of bacteria to growth is expected to be low due to their low numbers and near the critical food concentration for zero dark growth of *Ochromonas*. Triplicate cultures were kept semicontinuously, i.e. daily dilution to the initial cell density with medium in (a) and saturated bacterial suspension in (b) and (c), respectively. The density of the bacterial solution was kept constant by photometrical density measurements (750 nm).

Phagotrophic growth of *Ochromonas* did not deviate from mixotrophic growth (0.32 ±0.06 d^{-1} and 0.27 ±0.02 d^{-1}, respectively; μ ±SE). Phototrophic growth was nonsignificant (0.07 ±0.07 d^{-1}). Therefore, *Ochromonas* realized moderate growth by phagotrophic nutrition. Light potentially contribute to growth only at low food densities.

B. *Chlamydomonas* uses organics for growth at the DCM

We confirmed the inability of *Chlamydomonas* to grow only by photosynthesis at DCM using medium reflecting the lake's chemistry (1). At a PAR typically occurring at sunny days at the DCM (7 µmol photons $m^{-2}s^{-1}$) growth rates of *Chlamydomonas* without organics amounted to

–0.02 ±0.1 d^{-1} and to 0.25 ±0.03 d^{-1} with glucose addition (mean ±SD, semi-continuous culture experiments, 7.5 °C, 18 hours photoperiod).

ACKNOWLEDGEMENTS

We thank the staff from the UFZ and Potsdam University for processing the plankton samples and chemical analyses. U. Riebesell, G. F. Fussmann, K. O. Rothhaupt, J. Spindler, A. Kremp and W. Geller commented on the manuscript.

7 GENERAL DISCUSSION

The central aim of this thesis was to investigate the dominant phytoplanktonic alga, *Chlamydomonas* sp., from an extremely acidic mining lake (pH 2.7), to characterize the factors determining growth and use the findings to explain the vertical distribution of this alga in situ.

In order to conduct meaningful laboratory investigations, certain methodological pre-requisites had to be met. Firstly, I created a new medium, reflecting the mining lake's chemistry without addition of organic buffers, isolated the main planktonic organisms from the lake water and cultured them in the medium (Chapter 2). Another important pre-requisite was the simulation of the mining lake's light spectrum (shifted to higher wavelengths by the high iron content) in a specially developed experimental set-up (Chapter 3).

Once these methodological pre-requisites had been satisfactorily met, I studied the phototrophic growth of *Chlamydomonas* sp. under different concentrations of CO_2 in relation to light (μ/I relations), mixotrophic and osmotrophic growth with organic carbon in light and dark (Chapter 3), as well as its photosynthetic potential concerning inorganic and organic carbon supply and light conditions (P/I relationships, Chapter 4). Methods for the investigation of P limitation in single cells and its impact under the acidic conditions were tested (Chapter 5). Finally, in Chapter 6 the phenomenon of deep chlorophyll maxima (DCM), distinct in many of the lakes, was discussed and a new mechanistic theory for DCM formation presented with supporting evidence from laboratory and in situ experiments.

In this chapter, the results of Chapters 2-6 are summarized and integrated in explanations for the vertical distribution of the algae in Lake 111. The results are subsequently placed in a broader context and their ecological implications for the acidic mining lakes and the resolution of associated environmental problems in Lusatian region of Germany, discussed.

Factors influencing phytoplanktonic growth in acidic mining lakes: Explaining the vertical distribution and low cell densities of phytoplankton in Lake 111

In this thesis, I demonstrated that IC limitation can play a significant role in the upper water strata of acidic mining lakes in summer months, and is therefore likely to restrict growth of phototrophic algae. From the measured growth rates, it was possible to calculate the duration of IC limitation for the dominant green algae, *Chlamydomonas* sp., in different mining lakes (Chapter 3). The low *Chlamydomonas* cell densities realized in the epilimnion of Lake 111 can only partly be explained by IC limitation, because IC is continuously supplied from the atmosphere. Nevertheless, IC limitation in combination with the extreme ion concentrations present in the acidic mining lakes, enhances algal respiration rates (Chapter 4), implying that the algae face increased metabolic costs and subsequently their photosynthetic growth potential is reduced (Chapters 3 and 4). These metabolic costs may be one reason for the almost complete absence of purely autotrophic algae in the plankton of the acidic mining lakes (<10% of total biomass) and the dominance of specialized, mixotrophic flagellates such as *Chlamydomonas* sp. and *Ochromonas* sp.

Alkaline phosphatase activity suggesting phosphorus (P) depletion in individual *Chlamydomonas* cells varied spatially and temporally (Chapter 5). There was evidence that the realized cell densities in the epilimnion were lower than would be expected from the in situ SRP concentrations (Sas 1989). Under CO_2 limitation, the cells may exhibit an increased P demand, because *Chlamydomonas* sp. has the potential to induce a carbon concentrating mechanism (CCM) to deal with the extreme epilimnetic conditions. If so, this CCM would require increased amounts of energy in the form of ATP (Kaplan and Reinhold 1999). However, neither IC limitation nor P depletion could fully explain the vertical distribution of

Chlamydomonas sp. observed in Lake 111 which motivated investigations of other potential factors.

It is known from previous studies that some organisms can supplement their growth in extreme or non-extreme environments by combining nutritional strategies (Berninger et al. 1992, Laybourn-Parry et al. 2000). Such mixotrophy can offer them a competitive advantage over pure photo- or heterotrophs, because they can use light as energy source and combine it with use of organic carbon sources when light is limiting. Some mixotrophs supplement their energy, nutrient or vitamin supply by bacterivory (Caron et al. 1990, Thingstad et al. 1996, Hitchman & Jones 2000, Sanders et al. 2001). Purely phototrophic algae and purely heterotrophic flagellates play negligible roles in acidic mining lake plankton whereas the proportion of mixotrophs is extremely high (>90%, Nixdorf et al. 1998). The dominance of mixotrophy suggests that they have a competitive advantage potentially arising from a high metabolic flexibility of the mixotrophic strategy under the extreme physico-chemical conditions present in the acidic mining lakes when compared with circumneutral lakes of comparable trophic state. For example, in Lake Constance mixotrophs constituted only 23% of total summer algal biovolume (Gaedke 1998). The advantage of mixotrophy in other extreme environments, e.g. under conditions of light limitation (in deep water layers, in turbid reservoirs, in humic lakes, under snow and ice, at extremely high latitudes, or in dense algal populations due to shading), is presumably the more effective exploitation of limited resources (Berninger et al. 1992, Laybourn-Parry et al. 2000, Wetzel 2001). The mixotrophic strategy is also likely to be relevant in extremely acidic environments, such as Lake 111, given the poor underwater light climate. Bacterivory is unlikely to be relevant for our strain of *Chlamydomonas* because it was osmotrophic rather than phagotrophic and no evidence has ever been published for phagotrophy in this genus. However, in the deeper water strata of Lake 111, osmotrophic growth renders *Chlamydomonas* sp. competitively superior to pure

phototrophs, because DOC concentrations were higher than in the epilimnion (>10 mg L^{-1}) and light extremely low. By employing organic carbon in growth experiments, I showed that *Chlamydomonas* sp. has the potential to enhance its growth mixotrophically (Chapter 3). However, the vertical distribution of *Chlamydomonas* sp. in Lake 111 does not fully agree with vertical DOC concentrations (Fig. 6-3). It is likely that other factors apart from nutrient and carbon limitation influence the vertical distribution pattern observed, and Chapter 6 proceeded to elucidate another possible, novel mechanism to explain the formation of the DCM in Lake 111.

Previous studies have interpreted the formation of DCM as being the result of nutrient availability at depth (bottom-up control) (Carney et al. 1988, Letelier et al. 1993, Reynolds 1997, Gin et al. 1998, Klausmeier and Litchman 2001). Instead, the formation of DCM from a top-down perspective was investigated; studying the role of the grazers of *Chlamydomonas* sp. (Chapter 6). I showed that the second dominant phytoplanktonic alga in the acidic lakes, *Ochromonas* sp, exhibits primarily phagotrophic growth in illuminated strata, being able to feed on both, bacteria and *Chlamydomonas* sp. Phototrophy is inducible in the light during starvation periods and may be a long-term survival strategy for *Ochromonas* sp., as it is in the closely related species, *Poterioochromonas malhamensis* (Sanders et al. 1990). The data presented in Chapter 6 illustrate that the vertical distribution of *Chlamydomonas* sp. and *Ochromonas* sp. in Lake 111 may be attributed to the ability of the phagotrophic phototroph, *Ochromonas* sp., to graze its competitors for nutrients (*Chlamydomonas* sp. and bacteria) to densities below the food thresholds of larger grazers (i.e. ciliates in Lake 111) by combining phagotophy with phototrophic growth. In several investigations, the phagotrophic nutrition of *Ochromonas* spp. was shown to depend on light (Keller et al. 1994, Zhang and Watanabe 2001). In the DCM, light intensities limit the phototrophic growth of *Ochromonas* sp. and it is no longer able to maintain low *Chlamydomonas* sp. cell densities. This, coupled with the fact

that *Chlamydomonas* sp. utilizes DOC for growth, may lead to the pronounced accumulation of *Chlamydomonas* sp. cells, generating a DCM.

The results of this thesis illustrate the complex factors which influence the vertical distribution of phytoplankton in acidic mining lakes and influence the interplay between the few organisms forming the food-web in these lakes. The results presented in Chapters 2-6 all demonstrate the advantage of mixotrophic nutrition compared with purely autotrophic or heterotrophic nutrition under the extreme physico-chemical conditions and mixotrophs fill the niche generally occupied by pure auto- or heterotrophs (Azam et al. 1983, Weisse et al. 1990). All the higher organisms within these acidic environments (ciliates, heliozoans, rotifers) do not significantly contribute to biomass. The competitive advantage offered by mixotrophy is often favored in oligotrophic systems (Sieracki et al. 1993, Sommer et al. 1993, Laybourn-Parry et al. 2000).

Linking the results to the regional environmental and socio-economic impacts

As well as influencing the structure and functioning of the food webs in the acidic mining lakes, their extreme physico-chemical conditions cause immense hydrological and socio-economic problems for the former mining regions. For example, the mining activities and the flow of ground-, rain- and lake water through the surrounding landscape have contaminated prime agricultural land, lowering its pH and increasing the concentrations of heavy metals in the soils. Therefore, significant effort is being invested to develop different approaches to neutralize the lakes and render the area environmentally and economically viable.

In the Lusatian District, standard techniques, such as flooding with neutral river or ground water, are not possible due to a vast water deficit in the area (Nixdorf and Uhlmann 2002). Thus, new strategies are under development which focus on the bioremediation of the lakes.

A promising approach is biogenic-alkalinization: by adding organic substances and nutrients to the hypolimnion it is hoped to generate anaerobic conditions and stimulate microbial activity (Schultze et al. 1999). Sulfate- and iron-reducing bacteria are expected to reverse the process of pyrite oxidation, reducing the sulfate and iron to a stable state that can be locked in the sediments allowing the lake to naturally neutralize.

In situ enclosure experiments in Lake 111 are being employed to test the viability of such biogenic-alkalinization strategies. Initial findings suggest that the addition of organic waste materials such as straw does indeed stimulate bacterial activity. Interestingly, the addition of organic material also stimulated *Chlamydomonas* sp. blooms to develop in the surface strata of the water column (Fyson et al. 1998; Pöhler et al. 2002, Koschorreck et al. 2002), suggesting that i) the *Chlamydomonas* sp. were either exploiting the increased bacterial population, ii) or they were taking advantage of the organic carbon source, iii) and/or they were exploiting increases in inorganic nutrients such as P. The first scenario is highly unlikely since *Chlamydomonas* sp. has never been observed to ingest bacteria or fluorescently labeled particles. Scenarios ii) and iii) are in agreement with the findings from this study and imply that *Chlamydomonas* sp. is a potential competitor for bacteria and may influence the outcome of biogenic-alkalization. Therefore, the knowledge gained from this thesis about growth characteristics and limitation factors for *Chlamydomonas* sp. contributes to knowledge essential to predict the potential for algal development during alkalinization and the outcome of the bio-remediation strategies.

In a broader context, the results presented in this thesis have wider implications for many aquatic ecosystems. Chapter 6, in particular, presents a novel mechanistic explanation for the distribution patterns of phytoplankton observed in many oligotrophic oceans and lakes and, thus, challenges ecologists to rethink current paradigms.

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ACKNOWLEDGEMENTS

This study was sponsored by the BMBF (Bundesministerium für Bildung und Forschung) as part of the project entitled "Laboruntersuchungen zur Ökophysiologie von Planktonorganismen aus extrem sauren Tagebau-Seen" (UFZ 09/2000).

Sincere thanks to Prof. Dr. Ursula Gaedke for allowing me the scope to work independently, whilst always having an open door and open ears to discuss problems and results, and for her patient help with the revision of manuscripts and this thesis. I would also like to thank my second supervisor, Dr. Jörg Tittel, for his support with setting-up the experiments, his engagement and continuous supply of new ideas, as well as the critical and fruitful comments on my manuscripts and thesis. Without his organization and help the successful co-operation with the scientific team at the UFZ, Magdeburg, would not have been possible.

I am grateful to Dr. Peter Herzsprung and Dr. Robert Kringel for the chemical advice, as well as Dr. Gabriele Packroff and Barbara Zippel for discussions about plankton culturing and mining lakes processes. Close co-operation with Jörn Jander from the BTU Cottbus enabled the joint evaluation of the medium and isolation of the phytoplankton cultures. I want to thank Dr. Ingrid Chorus from the Institute for Water, Soil, and Air Pollution (WaBoLu) in Berlin for the opportunity to practice algal culturing in her laboratory and the kind help of her technical assistent Gabi Boettcher.

Elanor Bell patiently corrected my English and always motivated and supported me both scientifically and emotionally. I am most grateful for her invaluable and generous help.

Furthermore, I want to express my greatest thanks to my collegues Elly Spijkerman, Gregor Fußmann, Norbert Kamjunke, Antje Gerloff and Guntram Weithoff for their help and inspiring discussions regarding scientific and other questions. It is difficult to describe the manifold support Stefan Saumweber provided with computer and technical problems, but I

am sincerely grateful to him for everything. Last but not least, huge thanks to Ines Hübner, Sabine Donath, Christina Schirmer, Cornelia Hesse, Andrea Schmidtke and Sandy Tetsch for their support with sample measuring and culture work.