Biotechnological Aspects of Extracellular Polysaccharide Production by Microalga *Netrium digitus* using Twin-Layer

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Abstract

Netrium digitus is a representative of the species-rich class Zygnematophyceae (Streptophyta). Its intensive extracellular polysaccharide (EPS) production makes this alga interesting for biotechnological applications with focus on cosmetics and food additives. Quantitative data on growth and EPS production in suspension culture and for the first time in immobilized state using lab-scale porous substrate photobioreactors, so-called Twin-Layer (TL) systems, is provided in this thesis. Further, the influence of growth medium composition and culturing conditions was investigated. On the TL, cell dry weight of *N. digitus* increased linearly at a rate between 1.36 and maximally 5.01 $g \cdot m^{-2} \cdot d^{-1}$ and EPS dry weight followed an exponential development, reaching 10-30 $g \cdot m^{-2}$ after 20 days depending on the culturing conditions. Increased EPS production was observed, amongst others, at 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$ light intensity compared to 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$. Further increase was achieved by fivefold elevated nitrogen supply (25.02 $g \cdot m^{-2}$ EPS after 12 days) and pH decrease to 4.5 (21.88 $g \cdot m^{-2}$ day 11). Nitrogen depleted conditions and dilution of the medium to 1/4 of the original strength had a negative impact on ESP production. The analysis of the monosaccharide composition of the EPS showed xylose, fucose, glucuronic acid, an unidentified monosaccharide, galactose, glucose, rhamnose, mannose and galacturonic acid to be present in decreasing order. This order of abundance was not altered by any of the tested culture conditions, but differences in abundance of single monosaccharides were observed. E.g. TL EPS contained 33 % more fucose but 51 % less glucose than EPS from suspension culture. Fundamentals for immobilized culture of EPS producing microalgae are presented as well as suitable culturing conditions to increase production. Evidence of growth enhancement by the presence of bacteria in the algal culture is given as well as the introduction of an adapted spectrophotometric method for EPS quantification in culture supernatants using the inorganic dye Ruthenium Red.

Zusammenfassung

Netrium digitus ist eine Vertreterin der artenreichen Klasse der Zygnematophyceen (Streptophyta). Ihre hohe Produktion an extrazellulären Polysacchariden (EPS) macht sie für biotechnologische Anwendungen, insbesondere Kosmetik und Nahrungsergänzungsmittel, interessant. Diese Arbeit liefert quantitative Angaben zu Wachstum und EPS-Produktion in Suspensionskultur und erstmalig auch im immobilisierten Zustand unter der Verwendung von auf porösem Substrat basierenen Photobioreaktoren, sogenannten Twin-Layern (TL), im Labormaßstab. Desweiteren wurde der Einfluss der Nährmediumszusammensetzung und weiterer Kulturbedingungen untersucht. Auf dem TL stieg das Gewicht der Zellbiomasse mit einer Geschwindigkeit von 1.36 bis maximal 5.01 $g \cdot m^{-2} \cdot d^{-1}$ linear an und die EPS Produktion folgte einem exponentiellen Verlauf, wobei in Abhängigkeit der Kulturbedingungen 10-30 $g \cdot m^{-2}$ nach 20 Tagen erreicht wurden. Erhöhte EPS Produktion wurde unter anderem bei 330 µmol Photonen $\cdot m^{-2} \cdot s^{-1}$ Lichtintensität im Vergleich zu 70 µmol Photonen $\cdot m^{-2} \cdot s^{-1}$ beobachtet. Eine weitere Steigerung wurde durch Zugabe der fünffachen Stickstoffmenge (25.02 $g \cdot m^{-2}$ EPS nach 12 Tagen) oder Absenkung des pH-Werts auf 4.5 (21.88 $g \cdot m^{-2}$ an Tag 11) erreicht. Stickstofflimitierung und Verdünnung des Nährmediums auf 1/4 der Ausgangskonzentration hatten einen negativen Einfluss auf die EPS Produktion. Die Analyse der Monosaccharid-Zusammensetzung der EPS zeigte die Anwesenheit von Xylose, Fucose, Glukuronsäure, einer nicht-identifizierten Komponente, Galaktose, Glukose, Rhamnose, Mannose und Galakturonsäure in abnehmender Häufigkeit. Diese Reihenfolge änderte sich durch keine der verwendeten Kulturbedingungen, aber Änderungen in der Häufigkeit einzelner Monosaccharide wurden beobachtet. Zum Beispiel enthielten TL EPS 33 % mehr Fucose aber 51 % weniger Glukose als EPS aus Suspensionskultur. Grundlagen für die immobilisierte Kultur von EPS produzierenden Mikroalgen sowie geeignete Kulturbedingungen zur Erhöhung der EPS Produktion werden dargestellt. Belege der Wachstumssteigerung durch spezifische Bakterien in der Algenkultur sind aufgeführt, ebenso wie die Vorstellung einer adaptierten spektrophotometrischen Methode zur EPS Quantifizierung im Kulturüberstand unter Verwendung des anorganischen Farbstoffs Ruthenium Rot.

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List of Abbreviations

ACN acetonitrile

BSA Bovine Serum Albumin

BSM Bacterial Standard Medium

CCAC Culture Collection of Algae at the University of Cologne

CTAB cetyltrimethylammonium bromide

DSM Deutsche Sammlung von Mikroorganismen

EDTA Ethylendiamintetraacetic acid

EPS extracellular polysaccharides

HPLC high-performance liquid chromatography

mSFM modified Synthetic Freshwater Medium

OD optical density

PC polycarbonate

PCR polymerase chain reaction

PMP 3-methyl-1-phenyl-2-pyrazoline-5-one

PSA phenol-sulfuric acid assay

RR Ruthenium Red

SC sawascreen®

SDS sodium dodecyl sulfate

SFM Synthetic Freshwater Medium

TFA trifluoroacetic acid

TL Twin-Layer

UV ultraviolet

1. Introduction

Microalgae have gained increasing interest in the field of biotechnology in the past years. Focus is clearly on finding alternatives to fossil fuels (e.g. Chisti (2007); Brennan and Owende (2010)) but also interest towards high-value products has developed. Great potential is seen in the production of biochemicals for food, medicine, research and other uses by microalgae (Milledge, 2011; Borowitzka, 2013). These other uses are e.g. cosmetics for which bioactive, skin-friendly substances are of interest (Agatonovic-Kustrin and Morton, 2013). Applications as an excipient in the formulation or as an active agent are possible.

Well-known examples of ingredients produced by algae are agar, alginate and carrageenan (Jiao et al., 2011). The demand of these seaweed hydrocolloids has been stable over the past years, but unfortunately, shortages in seaweed material raised the production cost in the same time period (Bixler and Porse, 2011; Callaway, 2015). In addition, extraction of these polysaccharides includes alkali treatment as well as heating (Freile-Pelegín and Robledo, 1997), which are unfavorable techniques with regard to sustainable manufacturing processes.

Extracellular polysaccharides (EPS), produced by microalgae, can pose an addition to the portfolio of traditionally used algae-based polysaccharides or may even serve as a true alternative. As macroalgal hydrocolloids, EPS are also produced photoautotrophically and therefore only need solar energy along with inorganic nutrients and water for synthesis.

Certainly the chemical and physical properties of the EPS will differ from the traditionally used polysaccharides (Ha et al., 1988), but on the other hand, there is great potential for new bioactive properties that can be exploited. The investigation of extracellular polysaccharides further bears the advantage that energy intensive extraction protocols can be omitted because the polysaccharides are secreted by the cells.

The Zygnematophyceae, one of the ten major microalgal groups, includes many species with proportionally high EPS production. Biotechnological aspects of this potential source of polysaccharides are investigated using one *Netrium digitus* strain present in the Culture Collection of Algae at the University of Cologne

(CCAC), which was recognized for the remarkable gelatinous texture of the culture. The cultivation system of choice is the TL system, a porous substrate photobioreactor developed at the University of Cologne.
Culturing conditions such as light and nutrient supply were investigated and quantitative data on cellular biomass growth and EPS concentration were collected. In the course of this undertaking, a spectrophotometric method for EPS quantification was developed and positive influences of bacteria on zygnematophyceaen cultures were validated.

1.1 The Alga

Zygnematophyceae are freshwater organisms and closely related to land plants. New analyses even place them as the phylogenetic sister group of embryophytes (Turmel et al., 2007; Wickett et al., 2014). Within the Zygnematophyceae, the genus *Netrium* (Nägli) Itzigshon & Rothe (Fig. 1.1) forms its own lineage within the traditionally defined Zygnematales order (Gontcharov and Melkonian, 2010). Traditionally, this species is counted to the desmids, a heterogeneous group of unicellular and filamentous green algae, which can be further divided into saccoderm desmids, to which *Netrium* belongs, and placcoderm desmids, which include the Desmidiales such as the later discussed *Cosmarium* (Brook, 1981). *Netrium* cells are rod-shaped and contain two chloroplasts between which the nucleus is positioned (Brook, 1981). In contrast to members of the Desmidiales, the cell wall is single-layered, homogeneous and lacks pores. While many desmids are covered with a distinct mucilaginous sheath, the EPS secreted by *Netrium* in suspension culture slowly diffuses into the growth medium, causing the highly viscous texture.

Figure 1.1: *Netrium digitus* M3119

1.1.1 EPS Functions

Until now, focus of zygnematophyceaen research was on cell biology (e.g. Brosch-Salomon et al. (1998)), ecology (e.g. Coesel (1994)) as well as phylogeny (e.g. Gontcharov and Melkonian (2008)). Some studies considered the EPS and its possible functions with regard to the corresponding topic.
Many opinions concerning the function of the EPS for Zygnematophyceae have been announced so far. Some are based on experimental data. Others manifested themselves by repeatedly citing speculations and losing the speculative form at some point. Independent of their quality or availability of proof, the functions can be categorized into groups to simplify the overview.
Cell motility is well studied (Häder and Wenderoth, 1977; Häder, 1981, 1982) and evidence for the involvement of EPS secretion is given on several occasions (Domozych et al., 1993; Oertel et al., 2004). While the directed extrusion of mucilage at one pole of the cell can catapult the cell forward, this localized secretion is also used to adhere to substrates (Aubert et al., 1989) and to form networks with other cells (Surek and von Sengbusch, 1981).
Secondly, various protective functions are attributed to EPS. Extracellular substances often protect the incorporated microorganisms against desiccation (Boney, 1981). It was shown that a few desmids are able to survive drying in their vegetative state (Evans, 1958), but the influence of EPS in this matter was not clearly shown to date. Evidence is stronger concerning other Zygnematophyceae, such as *Zygnema*. It was demonstrated that its thick mucilaginous sheath protects the filamentous alga from water loss (Pichrtová et al., 2014).
Absorption of UV radiation by zygnematophyceaen EPS and high tolerance of the producing organisms against these short wavelengths (Boney, 1981; Lütz et al., 1997) is a verified correlation. Further, Coesel (1997) has shown that a mucilaginous sheath leads to protection against grazers because e.g. *Daphnia* discriminate against cells, which increase their biovolume by a sheath, compared to cells, where the EPS was removed by ultrasonication. Increased mucilage production at low temperature (4 ℃) was observed by Kattner et al. (1977).
The third field of EPS functions includes interactions with the surrounding environment. Pronounced mucilaginous sheaths around placcoderm desmids are commonly observed around species present in oligo- to mesotrophic waters while species in more eutrophic waters lack voluminous mucilage covers (Coesel, 1994). *Cosmarium abbreviatum*, found in an oligotrophic habitat, showed lower growth rates than the less mucilage producing *Staurastrum chaetoceras*, but a higher affinity to phosphate, when this nutrient was present at low concentrations

(Spijkerman and Coesel, 1996). Yeh and Gibor (1970) assumed that the mucilage serves as a trap for possibly scarce nutrients because they have observed a black deposit when protein together with Indian Ink was added to the mucilage of *Closterium acerosum*. While this speculation was taken up by many researchers and almost developed as validated by being repeatedly cited (e.g. Gouvêa et al. (2002); Giroldo et al. (2005); Piedras et al. (2010)), experimental prove stayed scarce.

Measurements with the electron paramagnetic resonance technique showed that spin labels with positively charged groups ($NH_3{}^+$) cannot pass the mucilaginous sheath of *Spondylosium panduriforme* and therefore cannot enter the cells (Freire-Nordi et al., 1998). Later also the calcium binding capacity of *Netrium digitus* mucilage and cell walls was demonstrated by electron energy loss spectroscopy in transmission electron microscopy (Eder and Lütz-Meindl, 2010). An increased number of mucilage vesicles in *Micrasterias denticulata* cells has been observed after addition of heavy metals as aluminum, zinc and copper (Volland et al., 2011).

An important ecological function of the mucilage is its use as a carbon source for other microorganisms. Closely associated bacterial communities were observed in the sheath but also on sheath-free desmids (Fisher and Wilcox, 1996; Fisher et al., 1998).

Further, EPS secretion is also part of the sexual reproduction cycle of *Netrium* (Biebel, 1964) and other desmids (Brook, 1981). It was also stated that the mucilaginous sheath increases the buoyancy of planktonic cells (Graham et al., 2008).

1.1.2 EPS Composition

Thinking about applications, the composition of the used EPS is of major importance. This is one of the reasons why research on the composition of commercially used macroalgal polysaccharides is far advanced compared to microalgal polysaccharides. Details about all present monosaccharides, the proportions of the anomers (α and β) as well as the position of linkages and sulfate residues are basic knowledge (Jiao et al., 2011). Variability in the quality of agar is known as well as that the red seaweed source is of major importance for the composition (Craigie et al., 1984).

This kind of information regarding microalgal polysaccharides is limited. Various researchers have investigated the monosaccharide compositions of desmidial EPS (e.g. Domozych et al. (1993); Lombardi et al. (1998); Surek (1982) and Kattner

et al. (1977)). Between seven (Domozych et al., 2005) to nine (Kiemle et al., 2007) monosaccharides were detected. Common features were xylose or fucose being the major component, higher quantities of galactose, glucuronic acid as well as minor abundance of glucose, mannose, arabinose, rhamnose, galacturonic acid and in some cases ribose. Differences between the investigated species were clearly present. Due to the high number of monosaccharides and therefore possible linkages, the outputs of linkage analyses were complex (Kiemle et al., 2007; Giroldo et al., 2005). Depending on the method of analysis, sulfate residues and methylated sugars were detected as well (Kiemle et al., 2007; Domozych et al., 2005).
Information on the variability in composition due to environmental factors is not available, but would be of interest for commercial applications.
Also of interest are possible biological activities of the polysaccharides such as the antibacterial effect by extracts from culture supernatants of *Cosmarium impressulum* (Söding et al., 1976), although the direct addition of bacteria to the growing *Cosmarium* culture did not reproduce this effect. Elsewhere, it was shown that the monosaccharide L-fucose suppresses allergic contact dermatitis (Hasegawa et al., 1980) and shows anti-aging effects (Péterszegi et al., 2003). Skincare products containing fucose as an active agent are commercially available (IMPAG Import GmbH, 2015).
In order to add to the knowledge, the monosaccharide composition of *Netrium digitus* EPS was investigated under different growth conditions.

1.2 The Culturing Technique

The culturing system selected for this study was developed at the University of Cologne in the work group of Prof. Dr. Melkonian. On this simple but promising porous substrate photobioreactor, microalgae are grown in an immobilized state and separated from the bulk medium. Depending on the application, the scale of the TL-system is adjustable. While microtiter scales are advantageous for high-throughput cultivation e.g. in collections (Nowack et al., 2005), larger scales can be used in pigment production (Benstein et al., 2014; Kiperstok et al., 2016), aquaculture feed production (Naumann et al., 2013) or wastewater treatment (Shi et al., 2007; Li et al., 2015).
One major advantage of this system lies in the aforementioned separation of biomass from the medium. It allows easy exchange of nutrient depleted medium for fresh medium or a medium with a different composition without disturbing the algae. In the case of EPS producing algae, this is of increased importance,

because centrifugation or filtration, conventional methods for separation of medium and cells, would also separate EPS from the cells. Further characteristics as high biomass concentration and utilization of high light intensities without photoinhibition (shown for *Halochlorella* and *Haematococcus*, Schultze et al. (2015); Kiperstok et al. (2016)) are additional advantages of the Twin-Layer.
Since the TL can be scaled up relatively easily, the production of EPS may be another possible application for this culturing technique.
During lab-scale immobilized cultivation of *Netrium digitus*, cellular biomass and EPS concentration were investigated under different culturing conditions and also compared to suspension culture, in order to evaluate the suitability of immobilized cultivation.

1.3 Interaction of Desmids and Bacteria

Organisms, living in the same habitat, inevitably interact with each other. These interactions include predator-prey relationships, parasitism, competition but also mutualism. As the pollination of flowers by insects has benefits for the plants as well as the animals, similar dynamics also take place at the microscopical level. An example, well known to botanists, involving microorganisms is the symbiosis of bacteria with plant roots (Kadereit et al., 2014). The close surrounding of the roots, therefore, is also termed "rhizosphere".
An equivalent zone can also be determined around microalgae. The immediate area around algal cells, with and without a mucilaginous sheath, is populated by other bacteria than the surrounding waters. Analogously to the rhizosphere, this area was named "phycosphere" (Bell and Mitchell, 1972). Due to the close proximity, the development of antagonistic as well as mutualistic and symbiotic relationships is logical (Knack et al., 2015). In fact, many reports clearly show growth promoting effects on algae by bacteria. These interactions include vitamin B12 production by bacteria (Croft et al., 2005), bacterial consumption of oxygen produced by algae (Mouget et al., 1995) and nutrient cycling (Currie, 1990).
When focusing on the main topic of this thesis, EPS production by desmids, knowledge about bacteria-algae interactions is of particular interest. The evaluation of possible losses of product due to degradation, and also positive influences by the bacteria, which might increase the EPS output, should be considered.
A positive relationship between the presence of bacteria, growth and possibly EPS production was evident by comparison of axenic with xenic cultures of

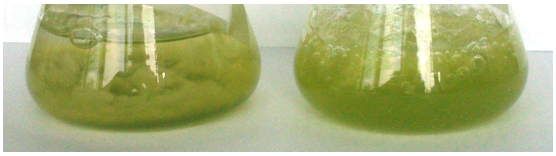


Figure 1.2: Axenic (left) and xenic (right) culture of *Cosmarium humile* after 5 months of growth at 16 °C and 15 µmol photons $\cdot m^{-2} \cdot s^{-1}$

several desmids (e.g. Fig. 1.2). Because a possible advantage of this effect can be used in biotechnological applications (q.v. Cho et al. (2014)), some growth dynamics of axenic and xenic cultures were recorded. In this case, not only *Netrium digitus* was investigated, but also the two *Cosmarium* species *C. humile* (M3051) and *C. pachydermum* (M2872), for which the effect was first observed and is particularly prominent.

1.4 Spectrophotometric Method for EPS Quantification

As the previous pages have made clear, the quantification of the EPS is of major importance for this work. Quantification of EPS in liquid samples is possible by drying a known volume by lyophilization (Domozych et al., 2005) or at high temperature. The necessary quantity of sample ranges from 15 to 90 mL of culture supernatant, depending on the amount of EPS. For an experimental set-up, calling for regular samples, cultures of at least 1 L are necessary in order to have sufficient material for all samples. If smaller cultures are desired, e.g. because many different conditions should be tested, an alternative method using smaller sample volumes is advantageous. Commonly, the phenol-sulfuric acid assay (PSA) by Dubois et al. (1956) is used for these cases (e.g. Kiemle et al. (2007) and Giroldo et al. (2005)). It is based on the reaction of phenol with the reducing ends of the monosaccharides, hydrolyzed from the polysaccharides by the added acid. Sample volumes of 1 mL are sufficient here, but the handling of highly hazardous substances may be positive to avoid. If a third, alternative method were available, it would be of particular interest if it were applicable in a microtiter format (total volume 200 µL) in order to save resources, time and minimize the risk of sample confusion.

This alternative may be a spectrophotometric assay using inorganic dye, which binds to acidic polymers. The so formed insoluble complexes can be removed from

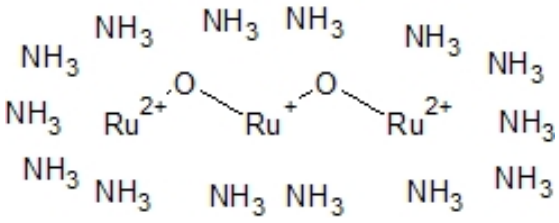


Figure 1.3: Structure of Ruthenium Red proposed by Fletcher et al. (1961)

the liquid by centrifugation and the absorbance of the supernatant is reduced according to the originally present polymer amount. A commonly used dye is Ruthenium Red (RR) with which a microtiter plate assay for EPS quantification was developed.

This polynuclear ionic complex, containing ruthenium and oxygen atoms and ammonia (Fig. 1.3), forms insoluble complexes with various polymers such as pectins and nucleic acids (Luft, 1971). In electron microscopy, RR is preferentially used to increase the density of cell walls and Golgi elements (Strycek et al., 1992). The first quantitative method using RR for polysaccharide determination was published by Figueroa and Silverstein (1989). RR solution was added to activated sludge pellets and the absorption of the supernatant after incubation was measured. Several variations of this method and also independently developed procedures can be found in literature (e.g. Hou et al. (1999) and Santhiya et al. (2001)).

A second field of use for the interaction of RR with polymers are enzyme activity assays e.g. for endo-polygalacturonases (Torres et al., 2011). In this assay, RR is used to quantify the reduction in pectin concentration. The activity of the enzyme that catalyzes the degradation of the pectin can be calculated from this value.

The use of RR with microalgal polysaccharides was investigated once before. In the study by Strycek et al. (1992) a diluted RR solution was incubated with cell suspensions and the absorption of the supernatant after centrifugation was measured. It was observed that RR staining increased with culture age, but it was not correlated quantitatively to EPS concentration.

This finding together with the knowledge about the quantitative sludge and pectinase assays give reasons that RR adsorption may be a good approach to quantify EPS. Therefore, an assay using RR and its complex forming properties was developed for quantification of EPS produced by zygnematophyceaen algae. The reduction to microtiter format as in Borucki et al. (2003) and Ortiz et al. (2014) was of particular interest.

2. Material and Methods

2.1 General Methods

2.1.1 Chemicals

All used chemicals are listed in Tab. 2.1.

Table 2.1: List of Chemicals

abbreviation/ formula	full name	purchased from	purity
	acetic acid	J.T. Baker	99-100 %
ACN	Acetonitrile ROTISOLV	Carl Roth GmbH	HPLC Gradient Grade (99.9 %)
	Agar Plant for cell culture	AppliChem	
NH_4Ac	Ammonium acetate	Merck	Ph Eur.
	Ampicillin Na-salt	ICN Biomedicals	
Ara	L(+)-Arabinose	Calbiochem	99.8 %
	Bio-Rad Protein Assay	Bio Rad	
$CaCl_2 \cdot 2\ H_2O$	Calcium chloride dihydrate	Carl Roth GmbH	$\geqq$ 99 %
	Chloroform	VWR chemicals prolabo	99.0 %
CTAB	cetyltrimethylammonium bromide	Aldrich	95 %
EDTA	Ethylendiamintetraacetic acid	Merck	molecular biology grade
Fuc	L(-)-Fucose	Alfa Aesa	99 %
Gal	D(+)-Galactose	AppliChem	pure Ph Eur
GalA	D-Galacturonic acid sodium salt	Fluka	$\geqq$ 98 %

..continued: List of chemicals

abbreviation/ formula	full name	purchased from	purity
Glc	D(+)-Glucose anhydrous	VWR chemicals prolabo	p.a.
	D(+)-Glucose monohydrate	Merck	for biochemistry
GlcN	D(+)-Glucosamine hydrochloride	Calbiochem	99.9 %
GlcA	D-Glucuronic acid sodium salt monohydrate	AppliChem	≧ 99 %
	HEPES	Carl Roth GmbH	≧ 99.5 %
	Iso-amyl-alcohol	Merck	p.a.
	Kanamycin monosulfate, USP	ICN Biomedicals	
$MgCl_2 \cdot 6\ H_2O$	Magnesium chloride hexahydrate	Merck	p.a.
$MgSO_4 \cdot 7\ H_2O$	Magnesium sulfate heptahydrate	Merck	p.a.
Man	D(+)-Mannose	Dr. Ehrenstorfer GmbH	99.5 %
	Meat extract dry	Merck	for microbiology
MeOH	Methanol HiPerSolv	VWR chemicals prolabo	HPLC Gradient Grade
PMP	3-Methyl-1-phenyl-2-pyrazoline-5-one	Aldrich	99 %
	Peptone from casein	AppliChem	
$K_2HPO_4 \cdot 3\ H_2O$	di-potassium hydrogen phosphate trihydrate	Merck	99.0 %
KNO_3	Potassium nitrate	Merck	99.0 %
Rha	L(-)-Rhamnose	Dr. Ehrenstorfer GmbH	98.3 %
Rib	D(-)-Ribose	Dr. Ehrenstorfer GmbH	≧ 99 %
RR	Ruthenium Red	Applichem	
NaCl	Sodium chloride	Merck	Ph. Eur.
SDS	sodium dodecyl sulfate	Serva	research grade
$NaH_2PO_4 \cdot H_2O$	Sodium dihydrogen phosphate monohydrate	Merck	p.a.
$NaHCO_3$	Sodium hydrogen carbonate	Merck	p. a.

..continued: List of chemicals

abbreviation/ formula	full name	purchased from	purity
$Na_2HPO_4 \cdot 2\ H_2O$	di-Sodium hydrogen phosphate dihydrate	Merck	p.a.
NaOH	Sodium hydroxide	Merck	p.a.
$NaNO_3$	Sodium nitrate	Merck	$\geqq$ 99.5 %
TFA	Trifluoroacetic acid	Fluka	$\geqq$ 99.5 %
Tris	Tris(hydroxylmethyl)-aminomethane	MP biomedicals	ultra pure
	Xanthan	Spinnrad GmbH	
	Yeast extract	Amresco	Ultra Pure Grade
Xyl	D(+)-Xylose	Supelco	99.9 %

2.1.2 Algal strains

The used algal strain M3119 *Netrium digitus* was provided by the CCAC and was made axenic by washing the culture with sterile Waris-H 3V medium (cf. Tab. 2.3) and centrifugation at 300 xg for five cycles before a cell suspension was sprayed on agar plates (Waris-H 3V with 1.5 % agar) by capillary force. Bacteria-free colonies were picked after two weeks of growth with a pipette tip and transferred into fresh sterile medium. Growth in BSM (cf. Tab.2.2) diluted 1:2, 1:9 and 1:99 in Waris-H 3V did not show any turbidity of the medium and confirmed the axenity.

For growth rate determinations with bacteria the strains *Cosmarium humile* M3051 and *Cosmarium pachydermum* M2872, both provided by the CCAC, were used. Stock cultures of the utilized algal strains were maintained in Waris-H 3V medium (*Netrium*) or Waris-H (*Cosmarium* strains) at 20 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (fluorescent lamps CoolWhite Lumilux 840 and Biolux 965, Philips, Hamburg, Germany) and 16 °C in 100 mL Erlenmeyer flasks containing 50 mL of growth medium. Transfer of ca. 1 mL of culture to fresh medium every 6 weeks assured viability.

Inoculum for experiments was grown in 2 L Erlenmeyer flasks containing 1 L of axenic culture and being aerated with sterile filtered air enriched with 1.5 % carbon dioxide. The temperature was stable at 23 °C and light intensity of 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ was regulated in a 14:10 light:dark cycle. Cultures being in late exponential to early stationary phase were used for inoculation of

experiments.

2.1.3 Growth Media

Waris-H and Waris-H 3V were used for stock culture maintenance and experimental cultures. The two media differ only in the volume of added vitamin solution (1 mL and 3 mL per liter, respectively). Further growth media used for the experiments were SFM and mSFM (Tab. 2.4 and 2.5). For all applications, including preparation of media, purified water (TKA, part of Thermo Scientific, www.thermofisher.com) was used.

Soil extract was produced by boiling 10 g garden soil for 10 min in 120 mL water, letting it cool, and filtering it consecutively through filters of decreasing pore size down to 0.2 µm. The clear filtrate was filled up to 100 mL and was stored at -20 °C until use.

The bacterial standard medium in Tab. 2.2 was also used in 1:1 diluted form and dissolved in Waris-H with additional 1.5 % agar for bacterial cultures (cf. Sec. 2.2.3).

Table 2.2: Composition of Bacterial Standard Medium (BSM)

Compound	weight per 1 L medium
Peptone	8.00 g
Glucose	1.00 g
Beef extract	1.00 g
Yeast extract	1.00 g

Table 2.3: Composition of Waris-H growth medium by McFadden and Melkonian (1986); for Waris-H 3V the threefold volume of vitamin solution was applied

Compound	final concentration	amount for 1 L stock solution	volume of stock solution per 1 L medium
KNO_3	1.00 mM	100.00 g	1.00 mL
$MgSO_4$ x 7 H_2O	81.10 µM	20.00 g	1.00 mL
$(NH_4)_2HPO_4$	0.15 mM	20.00 g	1.00 mL
$Ca(NO_3)_2$ x 4 H_2O	0.42 mM	100.00g	1.00 mL
HEPES	1.00 mM	238.13g	1.00 mL
PII-metals			1.00 mL
EDTA (Titriplex III)*	8.06 µM	3.00 g	
H_3BO_3	18.43 µM	1.14 g	
$MnCl_2$ x 4 H_2O	0.73 µM	144.00mg	
$ZnSO_4$ x 7 H_2O	73.00 nM	21.00 mg	
$CoCl_2$ x 6 H_2O	16.80 nM	4.00 mg	
Fe-EDTA			1.00 mL
EDTA (Titriplex II)*	17.86 µM	5.22 g	
$FeSO_4$ x 7 H_2O	17.90 µM	4.98 g	
KOH	1.00 M	54.00 mL	
Vitamins			1.00 mL
Vitamin B12	0.15 nM	0.20 mg	
Biotin	4.10 nM	1.00 mg	
Thiamine-HCl	0.30 µM	100.00mg	
Niacinamide	0.80 nM	0.10 mg	
soil extract			10.00 mL

Table 2.4: Composition of Synthetic Freshwater Medium (SFM)

Compound	final concentration	amount for 1 L stock solution	volume of stock solution per 1 L medium
$Ca(NO_3)_2$ x 4 H_2O	0.21 mM	100.00 g	0.50 mL
$MgSO_4$ x 7 H_2O	0.20 mM	20.00 g	2.50 mL
			0.60 mL
K_2HPO_4 x 3 H_2O	13.20 µM	5.00 g	
$NaNO_3$	0.35 mM	50.00 g	
Na_2CO_3	0.19 mM	32.00 g	
Vitamins			1.00 mL
Vitamin B12	0.15 nM	0.20 mg	
Biotin	4.10 nM	1.00 mg	
Thiamine-HCl	0.30 µM	100.00 mg	
Niacinamide	0.80 nM	0.10 mg	
HEPES	1.00 mM	238.13 g	1.00 mL
Tracemetals			1.00 mL
Na_2EDTA	14.92 µM	4.36 g	
$FeCl_3$ x 6 H_2O	11.65 µM	3.15 g	
primary trace metals			
K_2CrO_4	9.99 nM	19.40 mg	
$CoCl_2$ x 6 H_2O	42.03 nM	10.00 mg	
$CuSO_4$ x 5 H_2O	10.01 nM	2.50 mg	
$MnCl_2$ x 4 H_2O	0.91 µM	180.00mg	
Na_2MoO_4 x 2 H_2O	78.12 nM	18.90 mg	
$NiSO_4$ x 6 H_2O	10.27 nM	2.70 mg	
H_2SeO_3	10.08 nM	1.30 mg	
Na_3VO_4	10.00 nM	1.84 mg	
$ZnSO_4$ x 7 H_2O	102.12 nM	22.00 mg	

Table 2.5: Composition of modified Synthetic Freshwater Medium (mSFM)

Compound	final concentration	amount for 1 L stock solution	volume of stock solution per 1 L medium
$Ca(NO_3)_2$ x 4 H_2O	0.21 mM	100.00g	0.50 mL
$MgSO_4$ x 7 H_2O	0.20 mM	20.00 g	2.50 mL
			0.60 mL
K_2HPO_4 x 3 H_2O	92.25 µM	34.92 g	
KH_2PO_4	57.75 µM	13.16 g	
$NaNO_3$	0.35 mM	50.00 g	
Vitamins			1.00 mL
Vitamin B12	0.15 nM	0.20 mg	
Biotin	4.10 nM	1.00 mg	
Thiamine-HCl	0.30 µM	100.00mg	
Niacinamide	0.80 nM	0.10 mg	
Tracemetals			1.00 mL
Na_2EDTA		4.36 g	
$FeCl_3$ x 6 H_2O		3.15 g	
primary trace metals			
K_2CrO_4	9.99 nM	19.40 mg	
$CoCl_2$ x 6 H_2O	42.03 nM	10.00 mg	
$CuSO_4$ x 5 H_2O	10.01 nM	2.50 mg	
$MnCl_2$ x 4 H_2O	0.91 µM	180.00 mg	
Na_2MoO_4 x 2 H_2O	78.12 nM	18.90 mg	
$NiSO_4$ x 6 H_2O	10.27 nM	2.70 mg	
H_2SeO_3	10.08 nM	1.30 mg	
Na_3VO_4	10.00 nM	1.84 mg	
$ZnSO_4$ x 7 H_2O	102.12 nM	22.00 mg	

2.2 Experimental Set-up

2.2.1 Suspension Culture

Growth and EPS production in suspension culture was monitored over 24 days. For inoculation, cells from an aerated culture were isolated from the present mucilage by diluting the culture 1:1 with fresh sterile medium, shaking the mixture thoroughly for 1 min, centrifuging at 300 xg for 15 min and subsequently removing the supernatant containing the EPS and some remaining cells. The cell pellet was resuspended in a small volume of Waris-H 3V and the cell number of this suspension was determined by diluting an aliquot in a known volume of medium and counting 3 · 50 µL in wells of a 96-well-plate under an inverted microscope (Olympus CKX 41, Olympus Corporation, www.olympus-lifescience.com). Due to the large cell volume of *Netrium*, the use of a Neubauer chamber was dismissed. Aliquots of the dense algal solution were added to 1 L of sterile medium in 2 L Erlenmeyer flasks equipped with a 10 mL glass pipette for aeration with 1.5 % carbon dioxide and a magnet in order to stir the cultures prior to sampling. The initial cell number of the experimental cultures was adjusted to $500 \cdot mL^{-1}$.
The cultures were kept at 23 °C and 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ in a light:dark cycle of 14:10.
Harvest took place every three days and was performed under sterile conditions according to the following procedure: The cultures were homogenized by stirring on a magnetic stirrer for 5 min. Samples between 20 and 80 mL, according to the estimated cell density and EPS amount, were poured from the flasks into sterile centrifuge tubes and additional 1 mL aliquots were taken for cell counts. Cell counts were performed in 96-well-plates as described before. The large samples were diluted with sterile medium, if necessary, and shaken thoroughly for 1 min prior to centrifugation at 3000 xg for 15 min. Supernatant and pellet were separated and centrifugation of the supernatant was repeated, if cells were still visible within. The clear supernatant was frozen at -20 °C, lyophilized (Alpha 1-4 LSC freeze-dryer, Martin Christ Gefriertrocknungsanlagen GmbH, www.martinchrist.de) for 65 h and resuspended in 5 mL of water.

EPS Dry Weight Determination

Purification of the EPS was achieved by precipitation in four volumes of ethanol (96 %). The polysaccharides were allowed to settle overnight at 4 °C and were

collected by 15 min centrifugation at 3 000 xg and removal of the liquid. The precipitate was resuspended in water and frozen at -20 °C before lyophilization for 65 h. The weight of the dried EPS was determined by gravimetry.

2.2.2 Growth of *Netrium digitus* on Twin-Layer (TL)

TL System and Inoculation

Figure 2.1: Lab-scale TL system

For all presented data concerning the TL, lab-scale systems were used (Fig. 2.1). A detailed description of the system and the set-up can be found in Schultze et al. (2015). Light was provided either by fluorescent lamps (fluorescent lamps CoolWhite Lumilux® 840 and Biolux® 965, OSRAM GmbH, www.osram.de) at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ or by two sodium discharge lamps (SON-T AGRO 400W, Philips, www.lighting.philips.de) setting the light intensity to an average of 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$ or in one case to 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$. A light:dark cycle of 14:10 was applied. Carbon supply was realized by continuous aeration with pressured air enriched with 1.5 % carbon dioxide. Sawascreen® filter material (8054-61AF020106-08, 20 µm pore size, Sandler AG, www.sandler.de), cut to round filters of 25 mm diameter, as well as polycarbonate (PC) filters (0.4 µm pore size, 25 mm diameter, Nuclepore Track-Etched Membranes, GE Healthcare, www.gelifesciences.com) were used as substrate layer, on which the microalgae were inoculated.

Culture preparation for inoculation was similar to the suspension culture. A log phase *Netrium digitus* culture was harvested by diluting the culture with one volume of fresh medium, shaking and centrifugation at 300 xg. The cell pellets were collected and resuspended in fresh medium. The cell dry weight was determined by drying a known volume on pre-weighed filters for 1 h at 105 °C and subsequent weighing of the dry filter.

The density of the cell suspension was adjusted to 1.4 $g \cdot L^{-1}$ in order to achieve an inoculation density of approximately 5.5 $g \cdot m^{-2}$ when filtrating 1 mL on each filter (polycarbonate or sawascreen®). A filtration template assured the inoculation on a constant area of 2.54 cm^2 and dry cellulose tissues under the filter material provided the necessary suction for filtration.
In total, ten growth media were tested for *Netrium digitus* on the TL. The compositions of Waris-H 3V and mSFM pH=7 can be found in Tab. 2.3 and 2.5. Further variations of mSFM are shown in Tab. 2.6. Medium exchange was performed every 2-3 days along with every harvest, if not indicated otherwise.

Harvest and EPS Isolation

Harvest of at least three filters per condition was performed every 2-3 days. First, the biomass outside of the initial growth area was removed with the aid of a template ring. For the isolation of EPS from biomass, the biofilm was scraped off with a spatula and was placed in microreaction tubes containing fresh medium. Thicker biofilms were divided into multiple tubes. For fresh weight determination, pre-weighed tubes were used and total weight was determined. Repetitive mixing by pipetting resulted in a satisfactory separation of cells and EPS during centrifugation at 12 000 xg for 15 min. The supernatant was transferred to a fresh tube and the procedure was repeated. Not more than three centrifugations were necessary to separate the cells from the EPS. All cell pellets originating from one filter were combined with a small volume of medium and were filtered on the corresponding filter by vacuum filtration. The dry weight of the cell biomass was determined as described before (p. 17).
Similar to the determination in suspension culture, the EPS fraction was either stored at -20 °C or was directly precipitated in 4 volumes of 96 % ethanol. The precipitates were allowed to settle overnight at 4 °C and collected by centrifugation at 3 000 xg for 15 min. The then resuspended polysaccharides were frozen at -20 °C and lyophilized for 65 h. Subsequently, the dry weight of the EPS was determined gravimetrically.

Table 2.6: Variations of mSFM used for cultivation of *N. digitus* on TL with the specific conductivity; vitamins and tracemetal stock solutions are the same as in Tab. 2.5 and are given as mL stock solution per L medium, for the remaining components the final concentration is given

	mSFM pH=7	**mSFM pH=6**	**mSFM pH=6 non buffered**	**mSFM pH=4.5 non buffered**	**mSFM no N pH=6**	**mSFM +N pH=6**	**mSFM +NaCl pH=6**	**1/2 mSFM pH=6**	**1/4 mSFM pH=6**
					final concentration				
$Ca(NO_3)_2$ x 4 H_2O	0.21 mM	0.21 mM	0.21 mM	0.21 mM		0.21 mM	0.21 mM	105.00 µM	52.50 µM
$MgSO_4$ x 7 H_2O	0.203 mM	0.203 mM	0.203 mM	0.203 mM	0.203 mM	0.203 mM	0.203 mM	101.50 µM	50.75 µM
K_2HPO_4 x 3 H_2O	92.00 µM	19.80 µM	92.00 µM		19.80 µM	19.80 µM	19.80 µM	9.90 µM	4.95 µM
KH_2PO_4	58.00 µM	130.2 µM	58.00 µM	0.15 mM	130.2 µM	130.2 µM	130.2 µM	65.10 µM	32.55 µM
$NaNO_3$	0.35 mM	0.35 mM	0.35 mM	0.35 mM		3.43 mM	0.35 mM	175.00 µM	87.50 µM
$CaCl_2$ x $2H_2O$					0.21 mM				
NaCl							2.83 mM		
					mL of stock per L medium				
Vitamins	1	1	1	1	1	1	1	0.5	0.25
Tracemetals	1	1	1	1	1	1	1	0.5	0.25
			HCl addition to lower pH						
conductivity [µS·cm^{-1}]	179	173	180	173	132	532	532	86	43

2.2.3 Interaction of Desmids and Bacteria

Growth of two *Cosmarium* strains with and without Bacteria

The work was performed by Oliver Brumhard under the guidance of Alice Ekelhof.

In order to study the influence of bacteria on algal growth, bacteria from xenic cultures were isolated and re-inoculated to axenic cultures.
This approach was used for cultures of *Cosmarium humile* (M3051) and *Cosmarium pachydermum* (M2872), which were in their stationary phase (3 months old). The isolation was performed in a quantitative manner to also gain information on the algae-bacteria ratio. All steps were performed under sterile conditions with sterile equipment.
Cultures were homogenized by repeated pipetting and the algal cell number was determined using a Neubauer-counting chamber (Neubauer-improved, Paul Marienfeld GmbH & Co. KG, www.marienfeld-superior.com). Serial dilutions from 10^{-1} to 10^{-4} of the original culture were plated on Waris-H 1/2 BSM agar plates and then incubated for 4 days at 19 ℃. The bacterial strains were differentiated by optical selection. Colony counts allowed the calculation of a natural algal-bacterial ratio for every strain. Representatives of all distinguished bacterial strains were transferred to fresh agar plates and incubated under the aforementioned conditions and later stored at 4 ℃ and refreshed in liquid Waris-H 1/2 BSM before use.
Axenic *Cosmarium* cultures in their early stationary phase were liberated from their EPS by adding 5 % (v/v) 1 M KOH solution and incubating the mixture for 5 min. The culture medium including the EPS was removed after 10 min of centrifugation at 3 000 xg. This method was preferred compared to the otherwise necessary ultrasonication because sterility was insured. Cell counts and optical density (OD) measurements were preformed and the desired starting density of both organisms was adjusted in sterile centrifuge tubes with Waris-H. BSM was added to the axenic control cultures in order to exclude any effects by the transferred organics and nutrients. After overnight incubation, the suspensions were transferred to microtiter plates. Each combination of algal-bacterial culture was transferred into 60 wells of a microtiter plate, whereas the outer rows were only filled with sterile water to prevent evaporation of the culture medium. The plates were placed on transparent stands in water filled aquariums and were illuminated with 15 µmol photons $\cdot m^{-2} \cdot s^{-1}$ from the bottom.
Algal growth was monitored by direct cell counts in the culturing microtiter plate under an inverted microscope or by sampling a previously unsampled well and counting appropriate dilutions in wells of a fresh 96-well plate. Bacterial

density was determined by plating serial dilutions on Waris-H 1/2 BSM agar plates and counting the colony forming units after 3 days of incubation at 23 °C.

Growth of *Netrium digitus* in axenic and xenic Culture

Growth of xenic and axenic suspension cultures was monitored in Waris-H without vitamins nor soil extract, Waris-H with soil extract but no vitamins and standard SFM.
For inoculation, an axenic and a xenic culture in their stationary phase were diluted 1:1 with Waris-H without vitamins nor soil extract, shaken and centrifuged at 300 xg for 15 min. The supernatant was partially removed, leaving 10 mL for resuspension of the cell pellet. The cell numbers of the concentrates were determined and three 25 mL Erlenmeyer flasks per condition containing 12 mL of medium were inoculated with 0.9 mL of the xenic or axenic cell concentrates. The starting cell density of 410 cells per mL differed only by 1.75 % between axenic and xenic culture. Cultures were kept at 23 °C and 30 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (14:10 light:dark cycle) and were homogenized by repeated pipetting with sterile Pasteur pipettes prior to sampling. When necessary the cultures were diluted for counting. Five samples were taken over a period of 18 days.

Compositional Analysis of EPS Isolated from Axenic and Xenic Cultures

The work was performed by Oliver Brumhard under the guidance of Alice Ekelhof.

For the compositional analysis of EPS, produced in axenic and xenic cultures of *Cosmarium humile* and *Cosmarium pachydermum*, aerated suspension cultures (200 mL volume) were used. The cultures were grown at 23 °C, 90-100 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and with 1.3 % carbon dioxide in the aeration for seven weeks. For EPS isolation, the suspensions were treated with ultrasound in 40 mL aliquots for $3 \cdot 10$ sec on ice. Analogous to EPS isolation from *Netrium*, centrifugation and precipitation were performed (*q.v.* p. 16). The then freeze-dried samples were further processed as described in Sec. 2.3.3 (p. 25). The EPS concentration for hydrolysis was 8 mg$\cdot$mL^{-1}.

Identification of Bacterial Strains by 16S rRNA Gene Sequencing

The work was performed by Katharina Ziegler and Oliver Brumhard under the guidance of Alice Ekelhof.

To identify the seven bacterial strains, isolated from algal cultures, their DNA was isolated using the cetyltrimethylammonium bromide (CTAB) protocol by Doyle and Doyle (1987) and the 16S rRNA gene was sequenced.

For DNA isolation, bacterial strains were grown for 3 days in Waris-H 1/2 BSM at 27 ℃ and 200 rpm on an orbital shaker. Two milliliters of cell suspension of the respective strains were centrifuged (17 000 xg for 7 min) and the culture supernatant was discarded. Glass beads were added to the cells and the samples were placed into liquid nitrogen for a few minutes. To each sample 180 µL TE-buffer (10 mM Tris HCl pH 8; 1 mM EDTA) and 140 µL sodium dodecyl sulfate (SDS) 5 % were added. The samples were mixed and incubated at 55 ℃ for 20 min. Then 100 µL 5 M NaCl and 1 mL warm (65 ℃) CTAB-buffer (1.4 M NaCl; 100 mM Tris HCl pH 8; 25 mM EDTA, 2 % CTAB) were added. Mixing was followed by a 10 min incubation at 60 ℃ with shaking. After a 5 min centrifugation at 12 000 xg the supernatant was transferred to a new vessel and mixed with the same volume of chloroform:iso-amylalcohol (24:1). Incubation for 10 min in the dark was followed by 5 min of centrifugation (12 000 xg). The upper, aqueous phase was transferred and 0.66 volumes of 4 ℃ cold isopropanol were added. The mixture was inverted at few times and placed at -20 ℃ for at least 1 h. The precipitated DNA was collected by centrifugation at 15 000 xg for 15 min. The supernatant was removed and the pelleted DNA washed with 1 mL 80 % ethanol at -20 ℃ for 10 min and then centrifuged again. The washing step was repeated at least twice before the DNA pellet was dried and later resuspended in 50-200 µL TE-buffer.

Amplification of the 16S rRNA gene was achieved by polymerase chain reaction (PCR) using oligonucleotide primers SG_baci1 and SG_baci2 (Hess et al., 2016) and DreamTaq polymerase (Thermo Fisher Scientific Inc., www.thermoscientific.com) with its corresponding protocol and the thermocycler (Biometra,TProfessional basic, www.biometra.de) settings: 180 sec 95 ℃, 30 cycles of 45 sec 95 ℃, 60 sec 55 ℃and 180 sec at 72 ℃.

Successful PCR was verified on an agarose gel (1% agarose in 1x TAE buffer, Tab.2.7). PCR-products (3 µL) were mixed with 3 µL 5x Green GoTaq™ loading dye (Promega Corporation, www.promega.com) and loaded together with 1 kb DNA-Ladder (Thermo Scientific, www.thermoscientific.com). Thirty milliampere and 120 V were applied for 20 min.

Sequencing of PCR products was achieved using Dynabeads® M-280 (Invitrogen,

Table 2.7: Composition of 50x Tris-Acetate-EDTA buffer

Tris-Acetate-EDTA buffer	
Tris(hydroxymethyl)-aminomethane	242 g
0.5 M Na_2-EDTA	100 mL
Acetic acid	75.15 mL

Thermo Fisher Scientific, www.thermofisher.com) for purification and the primer pairs SeqH4F (IRD800-ATCTTRRGGTRGGCTTCCYAC) and Seq1040R (IRD700-ACTTAACCCRACATATCTCACGACACG) as well as Seq874F (IRD800-ACTCAAAGGAATTGACG) and SeqH49R (IRD700-TACGGCTACCTTGTTACGACTTC) for bidirectional sequencing reactions at 120 sec denaturation at 95 ℃, followed by 30 cycles of 30 sec 94 ℃, 30 sec 40 ℃ and 60 sec at 70 ℃. Gels were run on a Li-Cor IR^2 DNA sequencer (LI-COR Inc, www.licor.com).
The sequences were assembled and corrected using the AlignIR™ software (V2.0.48, LI-COR, www.licor.com). Strain identification was performed using BLAST Nucleotide Search (http://blast.ncbi.nlm.nih.gov) with the "minimal evolution tree" setting.

Antibiotic Properties of Xenic Algal Cultures

The work was performed by Till Barr under the guidance of Alice Ekelhof.

Xenic cultures of 15 zygnematophyceaen strains (Tab. 2.8) were grown under conditions described for the stock cultures (p. 11). Bacterial strains from each culture were isolated by plating on Waris-H 1/2 BSM with 1.5 % agar. All grown colonies were transferred to liquid Waris-H 1/2 BSM and grown for 24 h before plate diffusion tests were performed. Test organisms were *Bacillus subtilis* (DSM 347) and *Klebsiella trevisanii* (DSM 2688), which were grown at 30 ℃ and 140 rmp in Standard I (7.8 g·L^{-1} meat peptone, 7.8 g·L^{-1} peptone from caseine, 2.8 g·L^{-1} yeast extract, 5.6 g·L^{-1} NaCl, 1 g·L^{-1} D(+)-Glucose) and LB medium (10 g·L^{-1} tryptone, 5 g·L^{-1} yeast extract, 10 g·L^{-1} NaCl), respectively. *B. subtilis* was grown for three days to induce spore formation. Fifty milliliter of culture (OD=0.5) were added to 1 L of Standard I medium with 1.5 % agar at 60 ℃. After mixing, the solution was poured into petri dishes. Plates with LB-medium supplemented with 1.5 % agar were used to spread 100 µL of one day old *K. trevisanii* culture. Immediately after preparing the test organisms, sterile filter

disks dipped into algal or bacterial cultures were placed on the plates and were incubated for one week at 30 ℃. Filter disks with 25 µg·mL^{-1} to 1 mg·mL^{-1} kanamycin or ampicillin were applied as positive controls.
The diameter of the developed zones of inhibition was measured and the antibiotic strength, in terms of kanamycin/ampicillin equivalent, was calculated using a non-linear regression for the standards.

Table 2.8: Algal strains tested for antibiotic properties

M number	algal species	growth medium
M1366	*Planotaenium interruptum*	Waris-H
M1838	*Roya anglica*	Waris-H 3V
M1845	*Mesotaenium kramstai*	Waris-H
M2872	*Cosmarium pachydermum*	Waris-H
M3028	*Actinotaenium diplosporum*	Waris-H
M3031	*Cosmarium subtumidum*	Waris-H
M3049	*Cosmarium subprotumidum*	Waris-H
M3051	*Cosmarium humile*	Waris-H
M3107	*Cosmarium* sp.	Waris-H
M3119	*Netrium digitus*	Waris-H 3V
M3125	*Netrium* sp.	Waris-H 3V
M3166	*Netrium* sp.	Waris-H 3V
M3214	*Cosmarium* sp.	Waris-H
M3237	*Netrium* sp.	Waris-H 3V
M3354	*Micrasterias* cf. *fimbriata*	Waris-H

2.3 Analysis

2.3.1 Determination of Nutrient Concentration in Growth Medium

Nitrogen and phosphorus concentrations in the culture medium were determined with two spectrophotometric methods. To remove insoluble particles from the samples, centrifugation for 20 min at 17 000 xg was performed and only the supernatant was further processed.
The phosphorus measurement was adapted from Murphy and Riley (1962). One

liter of reagent was prepared by mixing 500 mL 2.5 M sulfuric acid, 150 mL ammonium molybdate (40 g·L^{-1}), 300 mL ascorbic acid (17.6 g·L^{-1}) and 50 mL of hydrated potassium antimony tartrate (2.86 g·L^{-1}). It was stored at -20 ℃ until use. For the measurement, 40 µL of reagent were added to 190 µL of water. Ten microliters of sample (culture supernatant) were mixed with the diluted reagent and incubated for 30 min at 40 ℃. After cooling and brief mixing, the absorbance at 882 nm was measured (Infinite® M2000, Tecan Trading AG, www.tecan.com). By also measuring sodium di-hydrogen phosphate standards from 0 to 0.2 mM the phosphorus concentration of the samples was calculated.
Remaining nitrate in the culture supernatant was determined with an assay by Miranda et al. (2001). Two hundred microliter of reagent (1 M HCl, 4 g·L^{-1} Vanadium(III)chloride, 5 g·L^{-1} sulfanilamide, 0.25 g·L^{-1} N-(1-naphthyl) ethylenediamine dihydrochloride) were added to 4 µL of sample and incubated at 40 ℃ for 30 min before the absorbance at 540 nm was measured. The nitrogen concentration was calculated using a standard curve between 0 and 3 mM $NaNO_3$.

2.3.2 Protein Concentration in EPS

Culture supernatants, collected during the suspension culture (cf. p. 16), were measured without further modification and also in concentrated form. Freeze drying and resuspending the samples in water allowed concentrations of 5 mg·mL^{-1} EPS or higher.
The protein content was determined using the BioRad Protein Assay (Bio-Rad Laboratories GmbH, www.bio-rad.com) according to the microtiter plate protocol described in the kit's manual. Bovine Serum Albumin (BSA), diluted to concentrations between 0.05 and 0.4 mg·mL was used as standard. Absorbance of the samples was measured at 595 nm with an Infinite® M2000 microtiter plate reader (Tecan Trading AG, www.tecan.com).

2.3.3 Monosaccharide Compositional Analysis

Dialysis and Hydrolysis

The freeze-dried EPS were resuspended in water at a concentration of 1 mg·mL^{-1} and then transferred to dialysis tubes (12-14 kDa MWCO, VISKING 20/32, Serva Electrophoresis GmbH, www.serva.de). Dialysis was carried out at 4 ℃ in 9 L of water, which was exchanged four times during 48 h. The EPS were

transferred to centrifuge tubes and frozen at -20 ℃ before lyophilization for 65 h. For hydrolysis an EPS concentration of 10 mg·mL^{-1} has proven to be favorable compared to lower concentrations. After the EPS were resuspended, the same volume of 8 M trifluoroacetic acid (TFA) was added. The tubes were sealed under a nitrogen stream in order to evacuate oxygen. For routine procedures, hydrolysis was performed at 110 ℃ for 4 h. Other hydrolysis conditions are indicated in the results section. After hydrolysis was completed, all liquid was removed by mild heating to 40 ℃ and a stream of nitrogen gas. Methanol (200 µL) was added and evaporated. This was repeated twice before the samples were redissolved in the same volume of water they were originally dissolved in for the hydrolysis.

Derivatization of Monosaccharides

To the hydrolyzed samples 10 % (v/v) glucosamine solution of known concentration was added. The samples and standards, both including internal standard, were derivatized following the protocol of Dai et al. (2010) with a few modifications.

A sample volume of 25 µL was mixed with the same volume of 0.6 M NaOH. Freshly prepared 3-methyl-1-phenyl-2-pyrazoline-5-one (PMP) (0.5 M in methanol, 50 µL) was added. The samples were incubated at 70 ℃ for 100 min with shaking at 300 rpm and were allowed to cool to room temperature before 50 µL of 0.3 M HCl was added. The proposed evaporation step (Dai et al., 2010) was omitted and 850 µL of 50 mM sodium phosphate buffer (pH=6.7) were directly added to the neutralized samples. After mixing, 400 µL of chloroform were added and the samples were vortexed. The chloroform was removed after the phase separation was complete and the procedure was repeated two more times. Finally, the samples were centrifuged at 8 000 rpm for 20 min and stored at 4 ℃ until they were injected into the HPLC.

Chromatographic Separation of Monosaccharides

For the quantitative analysis of the monosaccharide composition, a Merck-Hitachi L-6200 intelligent pump with Merck-Hitachi L-6400 UV-Vis detector set to 245 nm was used.

The derivatized samples were injected via a Rheodyne 7725i injector equipped with a 50 µL sample loop. Separation was achieved on a C-18 reversed

phase Spherisorb ODS-2 (250 x 4.0 mm, 5 µm particle size, Waters GmbH, www.waters.com) column and mobile phase A (10 % acetonitrile (ACN) in 50 mM sodium phosphate buffer pH=6.5) and mobile phase B (30 % ACN in 50 mM sodium phosphate buffer pH=6.5). The gradient used for routine analysis was the following:

time [min]	percent of A	percent of B
0	75	25
5	75	25
30	45	55

The column was re-equilibrated for 15 min before the next run was started. The flow rate was constant at 1 $mL \cdot min^{-1}$.
Recording of the detector output, calibration and calculation was achieved with Clarity software via Data Apex (Version 3.0.2.244, www.clarity-software.com and www.dataapex.com).

2.3.4 EPS Quantification with Ruthenium Red

A method for rapid EPS quantification by spectrometry was developed based on the reaction of Ruthenium Red (RR) with anionic polysaccharides.
For the larger reaction volumes, 0.1 mL sample was mixed with 0.9 mL RR solution in a reaction tube by vortexing. The mixture was then centrifuged at 4 000 xg for 15 min and the supernatant was transferred into a polyethylene cuvette. Absorbance was measured at 533 nm with a UV-2450 spectrophotometer (Shimadzu, www.shimadzu.com).
Down-scaling to a total volume of 200 µL changed the ratio to 20 µL sample and 180 µL RR solution. Mixing was accomplished by repeated pipetting. The microtiter plate, containing the mixtures, was centrifuged for 15 min at 3 000 xg and 80 µL of the supernatants were transferred into new wells. Absorbance measurement was performed at 533 nm with a Tecan Infinite M200 (Tecan Trading Group, www.tecan.com).
The RR solution was prepared by dissolving 1 $mg \cdot mL^{-1}$ of RR in water. It was stored at -20 ℃ until use. For the assay, the solution was diluted accordingly in order to achieve a final concentration of 12.6 $mg \cdot L^{-1}$.
The xanthan standards were prepared with xanthan gum powder in water between 0 and 300 $mg \cdot L^{-1}$. The algal EPS standards were produced by harvesting the corresponding culture in the stationary growth phase following the procedure described for the harvest of the suspension culture (p. 16). The supernatant was

Table 2.9: RR concentrations, pH of reagent solution and number of replicates measured for RR assay improvement

pH	RR conc. [mg/L]	number of replicates
3.00	117.20	3
4.32	38.52	3
4.32	195.88	3
7.50	5.93	3
7.50	117.20	10
7.50	228.47	3
10.68	38.52	3
10.68	195.88	3
12.00	117.20	3

concentrated to one quarter of the initial volume in a rotary evaporator at 50 ℃ and full vacuum before precipitation with ethanol took place. The cleaned EPS were lyophilized and a defined quantity was used for standard preparation in water.

For the screening of conditions, eight standard concentrations were measured per condition. The corresponding RR solutions were prepared by diluting the stock solution (1 mg·mL^{-1}) and adjusting the pH by the addition of the adequate volume of 0.1 M HCl and 0.1 M or 1 M NaOH, respectively. All tested concentrations and pH values are summarized in Tab. 2.9. Samples and working solutions were stored at -20 ℃ and were slowly thawed at room temperature prior to use.

2.3.5 Statistics

Curve fitting, as well as tests for significant differences (t-test or ANOVA), were performed with GraphPad Prism version 5.03 for Windows (GraphPad Software, www.graphpad.com). Differences were considered as significant when p-values were less or equal to 0.05. Curve fits to the exponential growth equation:

$$Y = Y_0 \cdot e^{(k \cdot t)} \tag{2.1}$$

are displayed as solid lines. Linear relationships

$$Y = Y_0 + slope \cdot x \tag{2.2}$$

are expressed as dashed lines. Second order polynomial fits

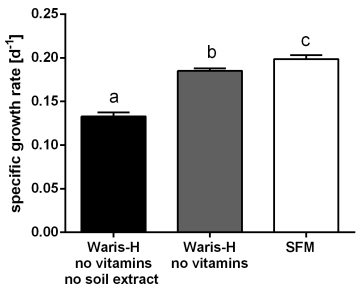
$$Y = B_0 + B_1 \cdot t + B_2 \cdot t^2 \tag{2.3}$$

are indicated by dotted lines and dotted-dashed lines represent third order polynomial fits.

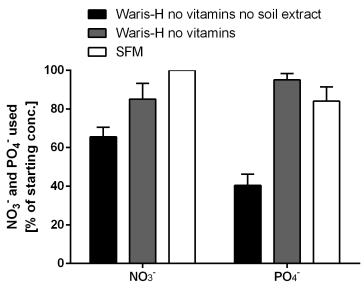
$$Y = B_0 + B_1 \cdot t + B_2 \cdot t^2 + B_3 \cdot t^3 \tag{2.4}$$

3. Results

3.1 Growth and EPS Production of *Netrium digitus* in Suspension



(a)



(b)

Figure 3.1: Growth of *N. digitus* in three different growth media, specific growth rate (a) and used nitrate and phosphate on day 14, presented as percent of the starting concentration (b), n=3

A preliminary media test with non-aerated cultures was performed with two variations of Waris-H as well as with standard SFM. Fig. 3.1a shows that all media are applicable, but with different results. The lowest growth rate (0.133 d^{-1}) was measured in Waris-H without any addition of vitamins nor soil extract. Vitamin addition led to an increase to 0.185 d^{-1}, which is still lower than the growth in SFM with 0.199 d^{-1}. It has to be noted that the microscopical observation of the cultures showed that the cells grown in SFM had a yellow, instead of a bright green, color and showed a reduction of the chloroplasts. To some extent, this was also true for cells in Waris-H without vitamins nor soil extract. The healthiest looking cells were the ones in Waris-H without vitamins.

In order to elucidate the reason for the growth differences the concentrations of the remaining nitrate and phosphate were analyzed (Fig. 3.1b). The algae in Waris-H without vitamins nor soil extract only used 65.49 % and 40.39 % of the available nitrate and phosphate, respectively. More uptake was observed in the culture without vitamins, where 85.07 % and 95.01 % of nitrate and phosphate, respectively, were removed from the culture medium. Complete depletion of

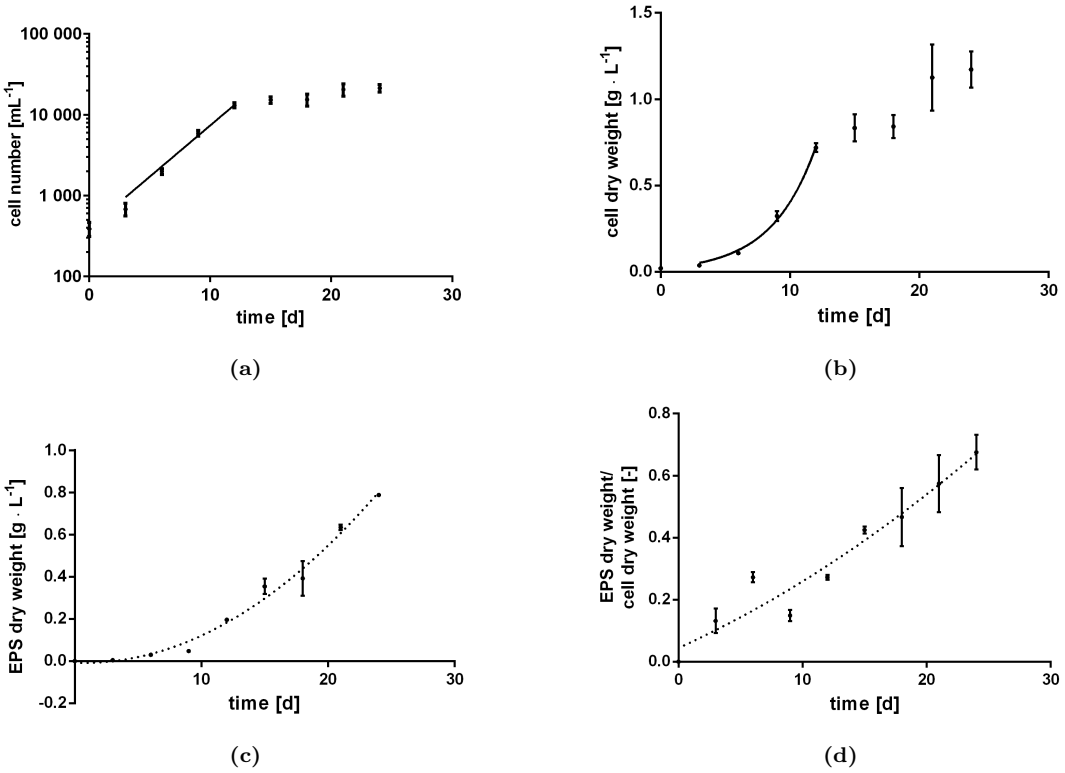


Figure 3.2: Suspension culture of *N. digitus* in Waris-H 3V at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and 1.5 % CO_2; cell number per mL (a), cell dry weight concentration (b), EPS dry weight concentration (c) and EPS dry weight per cell dry weight (d) over time, n=3

nitrate was achieved in the SFM culture. Here, 85.99 % of the phosphate was taken up.

Netrium and desmids, in general, are of interest for cell biological studies. They are less well studied regarding productivity of EPS and their growth characteristics. To fill this gap a classical suspension culture, where the mechanism of growth is known, interactions with bacteria can be precluded by a carefully maintained axenic system and more or less comparable literature can be found, was performed.

The increase of *Netrium digitus* cells in suspension culture (cf. Fig. 3.2a) follows a classical Monod growth kinetic (Monod, 1949). At first, the cells are in a short lag phase of not more than three days. The following exponential growth lasts for at least 9 days. Between day 3 and 12, the calculated doubling time was 2.37 d. After day 12, the stationary phase was reached and resulted in a final cell number of $2.14 \pm 0.022 \cdot 10^4$ mL^{-1}. The number of dead cells was also determined and never exceeded 5 %.

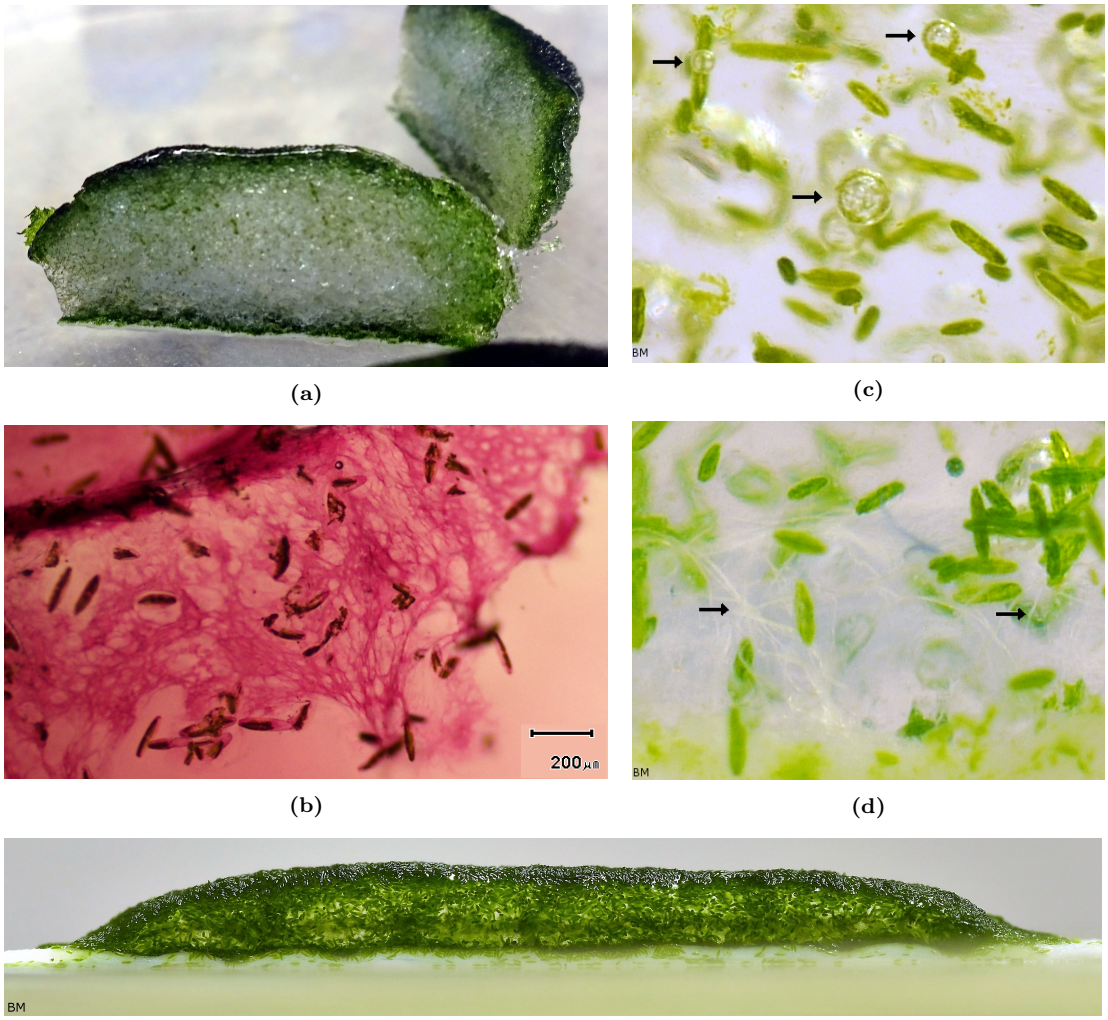
By using the determined average cell weight of 54.70 ng$\cdot cell^{-1}$, the dry weight of

cell biomass was calculated (Fig. 3.2b).
The EPS dry weight is shown in Fig. 3.2c. It must be emphasized that EPS production increases not only over time but also after day 12 when cell division already ceased. At the end of the experiment, an EPS concentration of 0.79 ± 0.003 g·L^{-1} was measured.
Because the development of cell number and EPS do not follow the same kinetic, comparison is made difficult. The calculation of the ratio of EPS per cell or per cell dry weight gives insight into the proportion of the two endpoints at each sampling point (Fig. 3.2d). This ratio also increased over time as the EPS concentration did. At day 24, 0.68 ± 0.05 g EPS were present per 1 g cell dry weight. This equals a percentage of 40 % EPS of the total biomass dry weight. The derivative of the curve in Fig. 3.2d describes the EPS productivity per unit of cell dry weight. In the case of the suspension culture, the productivity increased with time. The older the culture, the more EPS are produced by each cell, also after cell division had already ceased.

3.2 Growth and EPS Production of *Netrium digitus* on Twin-Layer

3.2.1 General Observations

For Twin-Layer (TL) cultivation, the microalgal cells are attached to a vertical substrate material layer, which separates the culture from the bulk of the supplied growth medium. Uptake of nutrients is not limited by this barrier and small substances, secreted by the algae, can enter the growth medium phase. This system was used for cultivation of *Netrium digitus* with the goal of EPS production. The alga readily grew in this immobilized state and also produced EPS. These polysaccharides stayed exclusively on the biofilm side of the system since no measurable amount of polysaccharides was recovered in the growth medium at any time of the cultivations.
When *Netrium digitus* was grown on TL, the intensive EPS production caused the biofilm to develop to large volumes. Usually, a thickness of 2 mm was already reached after 6 days at 330 µmol photons·m^{-2}·s^{-1}. Determination of the fresh weight of the biomass (Fig. 3.4) clearly illustrated this development. The maximum of 3.19 kg·m^{-2} in the displayed example corresponds to only 46.44 g·m^{-2} dry cell biomass and 20.76 g·m^{-2} dry EPS. Therefore, the percentage of dry matterwas only 2.11 %.



(a) **(c)** **(b)** **(d)** **(e)**

Figure 3.3: **(a)** 6 mm thick *Netrium digitus* biofilm grown on TL at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ for 27 days with Waris-H 3V, cut after freezing; **(b)** same biofilm stained with 5 mg$\cdot$mL^{-1} RR and excess stain washed off with water; **(c)** entrapped gas bubbles inside biofilm grown on TL at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ for 18 days with mSFM cut after freezing, **(d)** substrate material fibers extend into biofilm, same sample as (c); **(e)** biofilm grown on TL at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ for 29 days with Waris-H 3V without medium change, cut directly after harvest

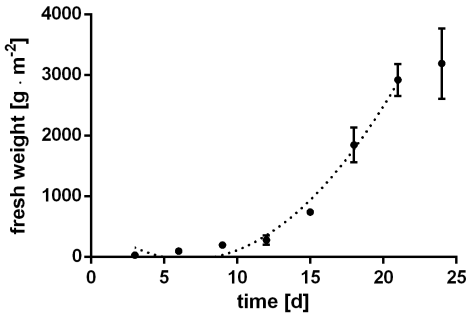


Figure 3.4: Fresh weight development of *Netrium digitus* biofilm over time grown at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ with Waris-H 3V, n=3

Depending on the conditions, the stability varied, and some biofilms lasted longer in the vertical position than others. The highest thickness reached was 10 mm after 20 days of culture, when in the last 14 days sodium chloride was added to the medium.

The culturing conditions influencing growth and EPS production will be elucidated in detail in the following sections.

A cut perpendicular to the filter surface revealed that the biofilm showed different layers (Fig. 3.3a). While the surface and the area close to the substrate material had an intensive green color, the inner part was almost colorless. This circumstance was confirmed by microscopy. On the surface, the cells were densely packed and preferentially orientated with their long side towards the light source (not shown). A similar situation was observed on the substrate material, which was covered with one to two layers of cells. When sawascreen® was used as substrate material, fibers were visible in the biofilm (Fig. 3.3d) and functioned as an additional support perpendicular to the growth surface. The inner part of the biofilm consisted mostly of mucilage, but cells, which had about two cell lengths of distance between each other, were also present. As staining with RR showed, the EPS forms an irregular, fibrous network (Fig. 3.3b), which is dense enough to entrap gas bubbles (cf. Fig. 3.3c).

3.2.2 Improvement of EPS Concentration on TL

After first TL cultivations with *Netrium digitus* had shown promising results, cultivation conditions were changed in order to improve the EPS yield.

In agreement with the suspension culture, immobilized cultures were grown at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ using fluorescent lamps. In order to evaluate the influence of the light source, sodium discharge lamps were tested as an alternative. Also, two substrate materials were tested. The cell dry weight development over time is displayed in Fig. 3.5a. In all cultures, the cell biomass

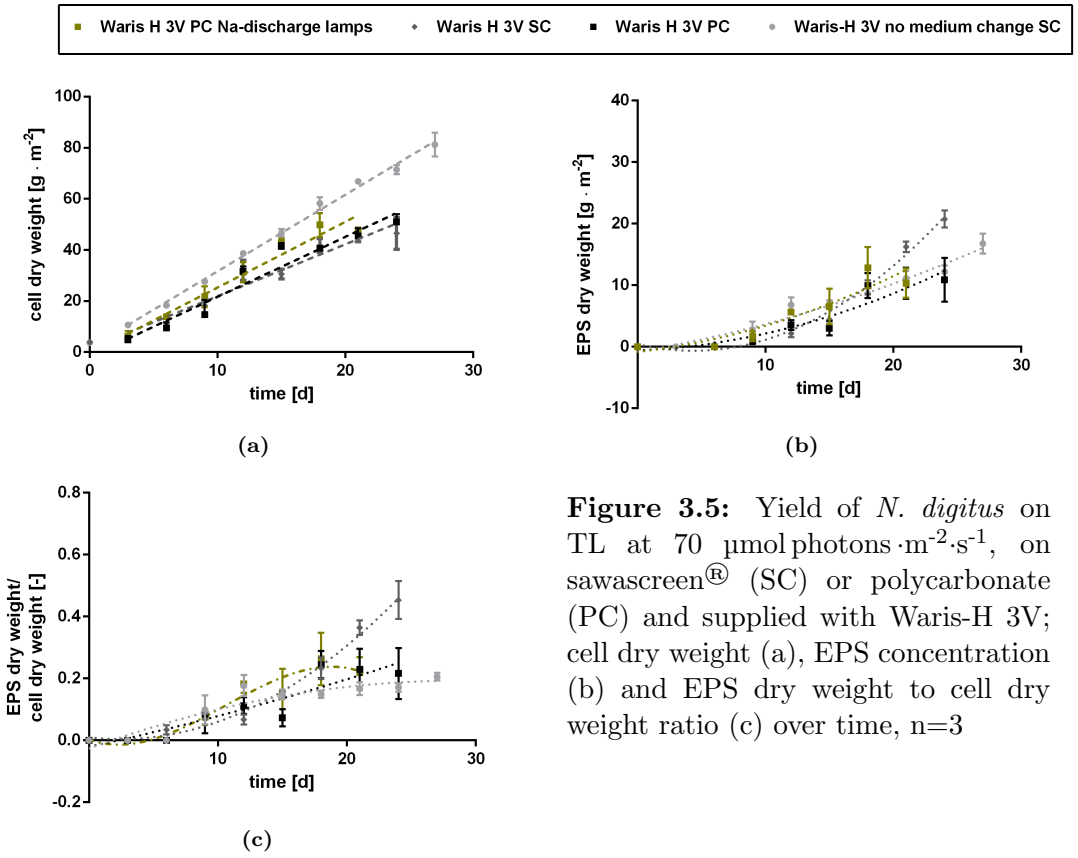


Figure 3.5: Yield of *N. digitus* on TL at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$, on sawascreen® (SC) or polycarbonate (PC) and supplied with Waris-H 3V; cell dry weight (a), EPS concentration (b) and EPS dry weight to cell dry weight ratio (c) over time, n=3

increased linearly. The average growth rate of the cellular biomass grown on PC and under sodium discharge lamps was 2.99 ± 0.14 g$\cdot m^{-2} \cdot d^{-1}$ until day 18. This is significantly higher than the growth of cultures illuminated by fluorescent lamps and supplied with fresh medium every three days. On PC filters and using fluorescent lamps only 2.34 ± 0.15 g$\cdot m^{-2} \cdot d^{-1}$ of cellular biomass were gained. The growth rate was even lower when sawascreen® filters were used (2.033 ± 0.10 g$\cdot m^{-2} \cdot d^{-1}$). When the medium was not exchanged and fluorescent lamps were used, *Netrium* grew at equal speed as with sodium discharge lamps (3.00 ± 0.06 g$\cdot m^{-2} \cdot d^{-1}$).

Fig. 3.5b shows the development of the corresponding EPS concentration over time. For all four conditions the first EPS were detected on day 9.

On SC material as substrate layer, a steep increase in EPS concentration was observed in the last six days (18-24) of culture. The final EPS concentration was 20.76 ± 1.25 g$\cdot m^{-2}$, which was 1.91 times more than on polycarbonate filters.

The two light sources were compared using polycarbonate filters. The EPS concentration developed in a similar way and the concentrations on day 21 were both 10.35 ± 2.06 g$\cdot m^{-2}$ for sodium discharge lamps and fluorescent lamps at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$. A steady increase in EPS concentration was observed when the growth medium Waris-H 3V was not exchanged for a period of 27 days.

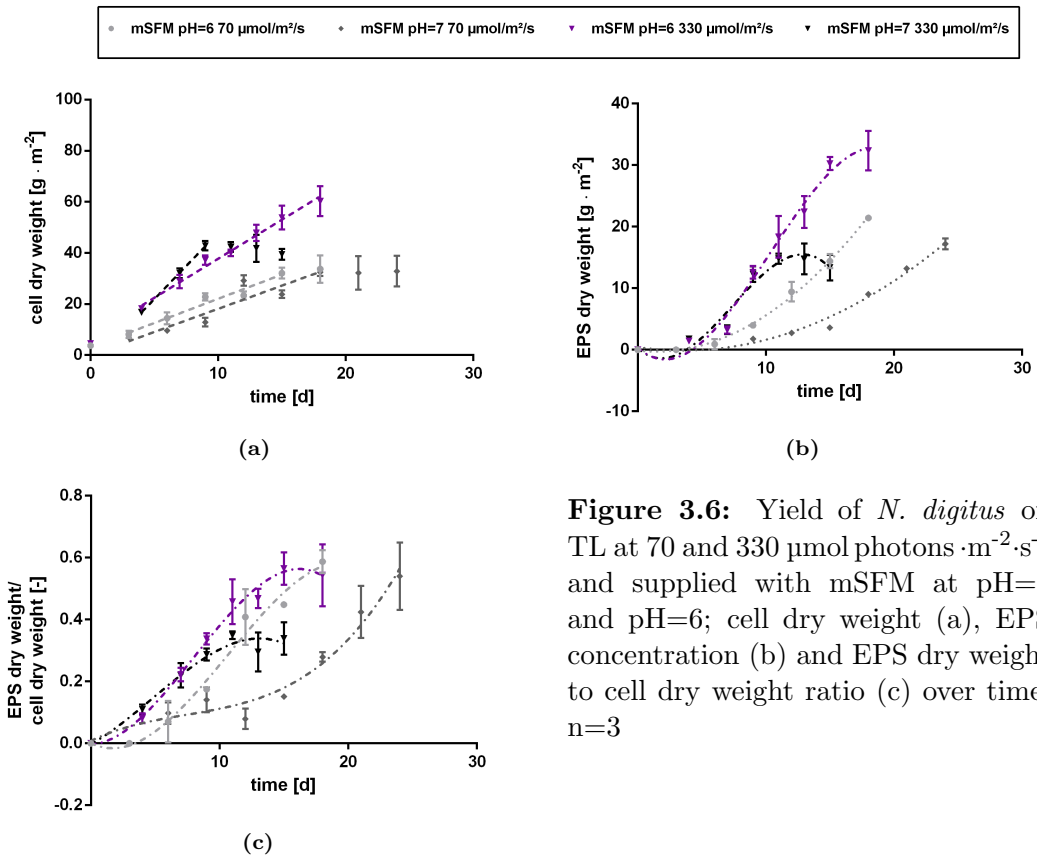


Figure 3.6: Yield of *N. digitus* on TL at 70 and 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and supplied with mSFM at pH=7 and pH=6; cell dry weight (a), EPS concentration (b) and EPS dry weight to cell dry weight ratio (c) over time, n=3

The final EPS concentration was 16.24 ± 1.34 g$\cdot m^{-2}$. A steeper increase during the end of the culture, as observed in the treatment with medium exchange and also on SC, was not observed.

Fig. 3.5c shows the calculated EPS per cell dry weight ratio for the just described experiments. The EPS amount per cell dry weight increased in all depicted experiments over time. While all conditions lead to a value between 0.07 (Waris-H 3V PC) and 0.16 (Waris-H 3V PC Na-discharge lamps) at day 15, the ratio developed differently from this time point on. For the sawascreen® material it increased to 0.46 ± 0.06 (day 24), and for the other experiments, the ratio did not exceed 0.27.

A newly developed growth medium, modified Synthetic Freshwater Medium (mSFM), was evaluated for *N. digitus* TL cultivation. At 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and with the pH set to 7 and 6 only 1.81 ± 0.15 g$\cdot m^{-2} \cdot d^{-1}$ and 1.90 ± 0.15 g$\cdot m^{-2} \cdot d^{-1}$ of cell dry weight were produced, respectively (Fig. 3.6a). This is not significantly different from the growth rate achieved in the respective experiment with Waris-H 3V (fluorescent lamps and SC). In contrast, a significant difference was seen in the EPS concentration. With mSFM at pH=7, comparable EPS concentrations as with Waris-H 3V were reached, but the decrease of the pH to 6 resulted in 21.42 ± 0.20 g EPS per square meter in only 18 days, which is 136 % more than

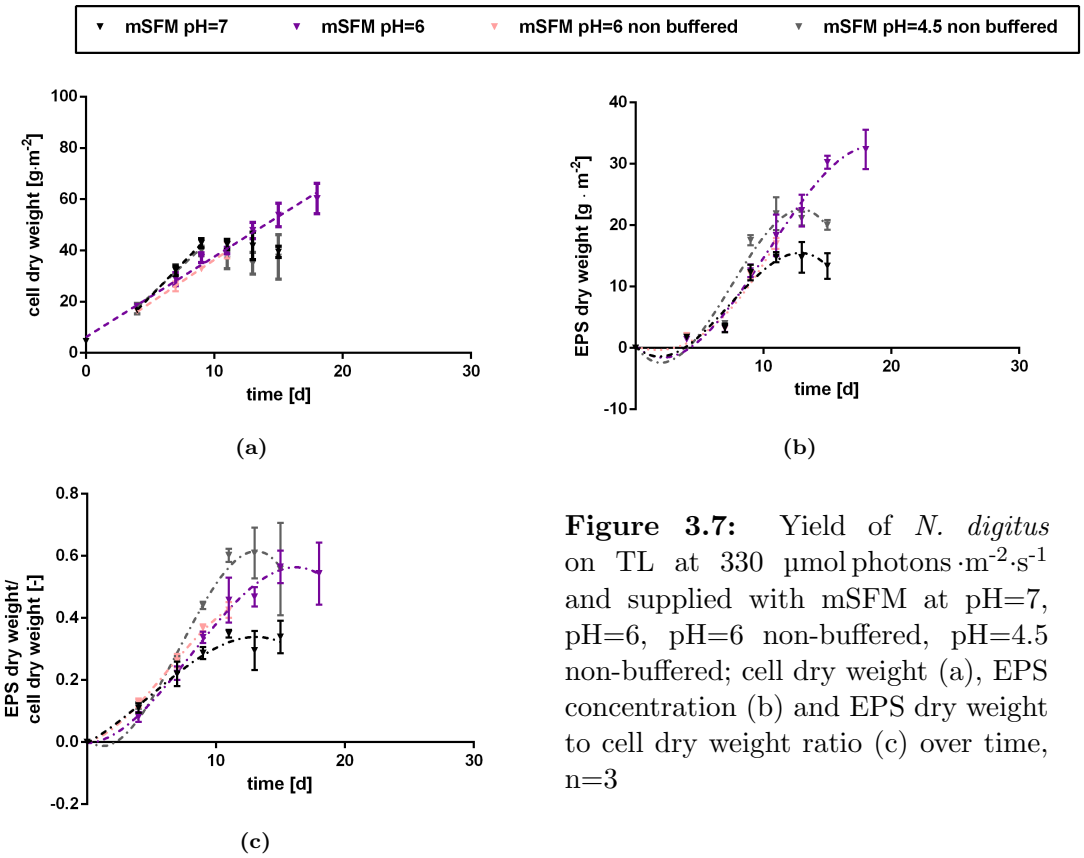


Figure 3.7: Yield of *N. digitus* on TL at 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and supplied with mSFM at pH=7, pH=6, pH=6 non-buffered, pH=4.5 non-buffered; cell dry weight (a), EPS concentration (b) and EPS dry weight to cell dry weight ratio (c) over time, n=3

at the higher pH at the same time point.

A higher light intensity of 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$ increased both, cellular biomass and EPS concentration. At 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$, growth rates of 5.18 ± 0.21 g$\cdot m^{-2} \cdot d^{-1}$ and 3.06 ± 0.13 g$\cdot m^{-2} \cdot d^{-1}$ were measured for mSFM with a pH of 7 and 6, respectively. These growth rates correspond only to the time period of linear increasing biomass. At high light and pH=7 no further increase in cellular biomass was observed after day 11, a time at which the biofilm already had developed a thickness of approx. 5 mm. At low light intensity and pH=7 growth proceeded until day 18 before a stagnation was observed. For a pH of 6 the linear period was longer at high light intensity and lasted until the end of the cultivation on day 18. The EPS concentrations developed in a similar fashion as the cellular dry weight. On day 11, the EPS concentrations were already 14.80 ± 0.82 g$\cdot m^{-2}$ and 18.34 ± 3.39 g$\cdot m^{-2}$ for mSFM pH=7 and pH=6 (both 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$), respectively. In the following days no big increase was detected for pH=7 anymore. In contrast, *N. digitus* grown with mSFM at pH=6 showed further increase in EPS per area until the end of the culture at day 18 and produced 32.36 ± 2.61 g $\cdot m^{-2}$ EPS.

The EPS per cell dry weight ratios over time are shown in Fig.3.6c. In the first half of the cultivation, more EPS were present per cell dry weight unit

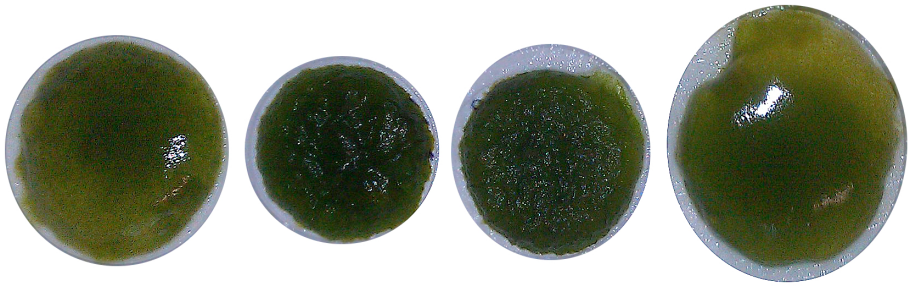


Figure 3.8: The effect of pH on the biofilm development can be seen by comparing the thickness and the color of the *Netrium digitus* biofilms grown with (from left to right) mSFM at pH=7, pH=6, non-buffered pH=6 and pH=4.5 for 11 days

under high light conditions than under low light. Further, it can be seen, that the development of the two cultures supplied with mSFM pH=6 was parallel except for the last sampling point. Similar final EPS/cell dry weight ratios were reached for the two mSFM pH=6 cultures (0.59 ± 0.02 and 0.56 ± 0.04 for low and high light, respectively) and the culture grown at low light and with mSFM at pH=7 (0.54 ± 0.10). At high light and pH=7 only 0.34 ± 0.04 g EPS per g cell dry weight were reached (day 15).

At high light two more variations of the pH in mSFM were applied (Fig. 3.7). The results for medium with pH=7 and pH=6 were already explained in the previous paragraphs and are added for easier comparison.

The exhaustion of the buffer capacity at pH=6, before the medium was used, did not have any effect on growth nor EPS concentration. The growth rate of 3.37 ± 0.16 g·m^{-2}·d^{-1} was not significantly different to the one with medium with buffer capacity and the EPS concentrations were also equivalent.

The cellular biomass grown with mSFM pH=4.5 increased at 4.68 ± 0.27 g·m^{-2}·d^{-1} until day 9. This growth rate is equal to growth at pH=7 but significantly higher than at pH=6. Comparable to mSFM pH=7, the biofilm at this low pH had also developed a thickness of 5 mm when growth ceased around day 9. The EPS concentration increased at a higher rate than in the other cultures and reached 21.88 ± 2.18 g·m^{-2} after only 11 days. After this time point, no further increase was observed, and the cultivation ended after day 15 because the biofilm slid off the surface.

A clear trend can be seen when the ratios of EPS and cell dry weight are considered (Fig.3.7c). The lower the pH, the higher the EPS per cell dry weight. As a result, the highest ratio of all TL experiments was measured on day 11 with mSFM pH=4.5: 0.61 ± 0.07.

The effect of pH on the biofilm development can also be seen by comparing the thickness and the color of the *N. digitus* biofilms grown on mSFM with pH of

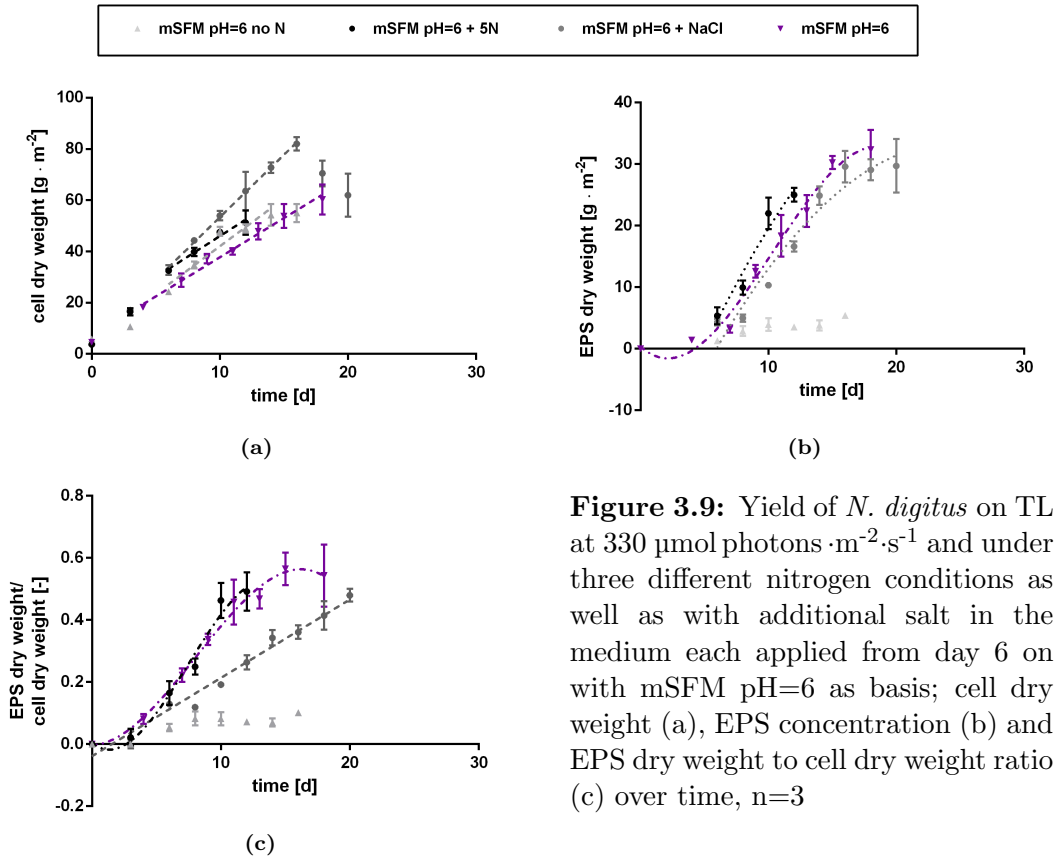


Figure 3.9: Yield of *N. digitus* on TL at 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and under three different nitrogen conditions as well as with additional salt in the medium each applied from day 6 on with mSFM pH=6 as basis; cell dry weight (a), EPS concentration (b) and EPS dry weight to cell dry weight ratio (c) over time, n=3

7 to 4.5 (Fig. 3.8). The biofilms grown at pH=7 and pH=4.5 are considerably thicker, and their color is lighter than the biofilms grown at pH=6. While the biofilm at pH=4.5 had a high EPS concentration, the one with pH=7 had the lowest EPS concentration of the four conditions, even though it was thicker than the ones at pH=6.

The satisfactory EPS concentrations, achieved using mSFM pH=6, together with the increased stability of the biofilm compared to the other pH settings led to the use of this medium for further cultivations. In these, stress conditions were applied in order to increase the EPS concentration further. All stress experiments were conducted at high light intensity, whereas some components or the strength of the medium were changed after 6 days of culture. The growth rates were therefore only calculated from this time point on.

Omitting the nitrogen source in mSFM after 6 days of growth did not result in a decrease in growth rate within the first 8 days of this stress (Fig. 3.9a). The growth rate was calculated to be 3.71 ± 0.36 g$\cdot m^{-2} \cdot d^{-1}$. Only on day 16 no further increase in cellular biomass was measured anymore. Nevertheless, microscopical observation on day 12 already showed many cells with enlarged vacuoles and of brown color (Fig. 3.10). In contrast to the cultures in which growth also ceased (e.g. high light and pH=7), this biofilm was much thinner. Only 1.5 mm

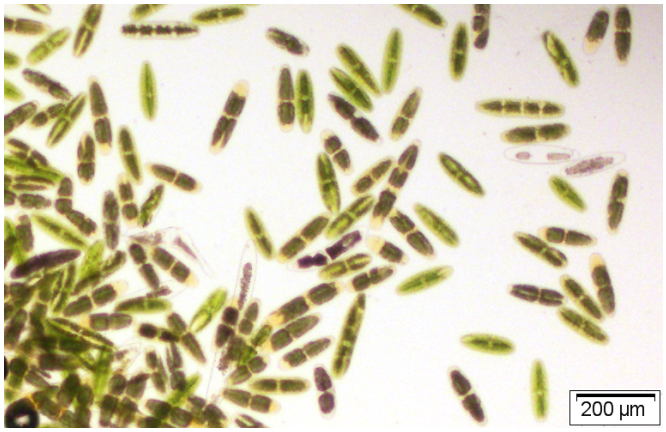


Figure 3.10: *N. digitus* cells grown on TL supplied with mSFM without nitrogen for 6 days at 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$

were measured on day 16. The measured EPS concentrations are consistent with this observation. The nitrogen limitation from day 6 on caused the EPS concentration to stabilize at the low value of 5.44 ± 0.25 g$\cdot$m^{-2}.
The addition of 5-fold nitrogen led to a growth rate of 3.20 ± 0.33 g$\cdot$m$^{-2}\cdot$d^{-1}. This was not significantly different from the control nor the nitrogen limitation treatment. In contrast to these biofilms, nitrogen addition resulted in a thick biofilm, which slid off the surface after day 12. The EPS concentration of this biofilm was 25.02 ± 0.93 g$\cdot$m^{-2} at this time point, corresponding to a gain of 18.69 % compared to mSFM pH=6 at day 12.
The addition of nitrogen inevitably leads to an increase of the medium's conductivity. To have a comparison for this conductivity increase, *Netrium digitus* was also cultured with mSFM containing the normal nitrogen amount but additional salt (NaCl) and therefore the identical conductivity of 532 µS$\cdot$cm^{-1} as the high nitrogen medium. With 4.87 ± 0.22 g$\cdot$m$^{-2}\cdot$d^{-1}, this conductivity control showed increased growth until day 16. After this time point, the biomass concentration decreased, but the biofilm thickness increased further. With maximally 10 mm the highest thickness of all treatments was measured on day 20. The EPS concentration followed the same pattern as the control (mSFM pH=6) and reached a maximum of 29.74 ± 3.55 g$\cdot$m^{-2} on day 16. After this time point, the concentration stayed stable.
The ratio of EPS dry weight and the cell dry weight is particularly interesting for this set of experiments. The addition of nitrogen resulted in the same pattern as most high light cultures, at first a fast increase of the EPS per cell biomass ratio followed by stabilization. The final ratio of 0.49 ± 0.05 was comparable to the control (mSFM pH=6) at the same time. The high cell growth rate in the +NaCl

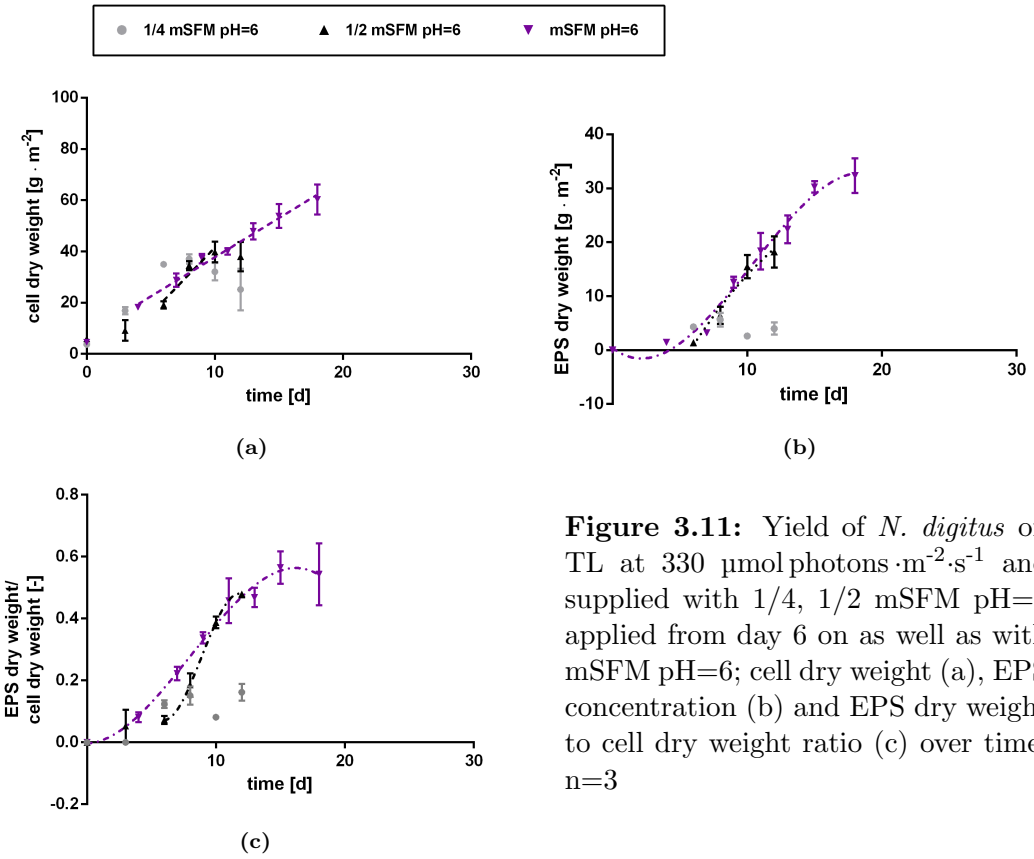


Figure 3.11: Yield of *N. digitus* on TL at 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and supplied with 1/4, 1/2 mSFM pH=6 applied from day 6 on as well as with mSFM pH=6; cell dry weight (a), EPS concentration (b) and EPS dry weight to cell dry weight ratio (c) over time, n=3

culture counteracted the average EPS concentrations resulting in a comparably low EPS per cell biomass ratio of 0.26 ± 0.02 on day 12. In contrast to others, the increase in this treatment is best described by a linear function. On day 20, a ratio of 0.48 ± 0.02 was reached. In the nitrogen limited condition, growth of cell biomass ceased after day 14, together with the minimally increasing EPS concentration the ratio of the two barely exceeded 0.10.

As can be seen in Fig. 3.11a, the dilution of the growth medium had an effect on growth. Compared to the control with full strength medium, growth was increased at half strength mSFM. Between day 6 and 12 cellular biomass increased at 5.17 ± 0.74 g$\cdot m^{-2} \cdot d^{-1}$ compared to 3.06 ± 0.13 g$\cdot m^{-2} \cdot d^{-1}$ with 1/2 mSFM pH=6 and mSFM pH=6, respectively. The EPS concentration was not affected by the dilution. It was at 18.21 ± 2.37 g$\cdot m^{-2}$ at the last harvesting point on day 12. Consequently, the ratio of EPS and cell dry weight differed a little more, being lower on day 6 and 8 for 1/2 mSFM but later reaching the same value (e.g. 0.48 ± 0.01 on day 12) as the control and the high nitrogen condition. Further dilution to 1/4 of the original strength had a negative effect. No increase in cellular biomass was measured after the change of medium on day 6. Furthermore, the green color of the biofilm faded and the consistency got more liquid. As a consequence, the biomass spread over a larger surface, which resulted

in a negative growth rate of -1.72 ± 0.62 $g \cdot m^{-2} \cdot d^{-1}$. The EPS concentration was close to a stable value of 4.00 ± 0.91 $g \cdot m^{-2}$. The EPS/cell dry weight ratio never exceeded 0.16. After day 12, harvest was not possible anymore because the whole biofilm had slid off the filter.

From the presented results on TL cultivation, some preferential conditions can be deducted.

Highest growth rates were measured at high light intensity with mSFM pH=7 and pH=4.5 as well as 1/2 mSFM pH=6. In these cases, the linear growth period was short and only lasted until day 9 and 10, respectively. The longest linear growth period of 18 days was observed at high light with mSFM pH=6 and 27 days at low light and no change of Waris-H 3V.

Analogous to cellular biomass, EPS concentrations were also higher at high light intensity. At low light, the highest measured EPS concentration was 21.42 $g \cdot m^{-2}$ on day 18 in the treatment with mSFM pH=6. Comparable amounts were already measured 6 days earlier under high light conditions. Variation of the medium at these light conditions showed that nitrogen depletion, as well as dilution of the medium to 1/4 mSFM (pH=6), had a negative effect on EPS production. On the other hand, decreasing the pH to 4.5 and addition of nitrogen resulted in an increase of EPS per area.

3.3 Composition of *Netrium digitus*' EPS

3.3.1 Methodology

It is well known that the extracellular polymeric substances of microalgae mostly consist of polysaccharides. For the analysis of the monosaccharide composition of *Netrium digitus*' EPS several treatment steps were necessary. First, the polysaccharides were separated from the algal cells and cleaned from other contaminations. Second, the polysaccharide chains were cleaved to monosaccharides. These were then separated and quantified by chromatography. Since the evaluation of hydrolysis results is only possible if a separation method of the monosaccharides was established before, the steps of the chain of treatments have to be implemented in the reversed order of their application. In the following, the improvements of the analysis are presented in the order they were implemented.

HPLC Conditions

Separation and subsequent detection of eight neutral sugars and two uronic acids was achieved on a C18 reversed phase Sherisorb ODS2 column. Suitable separation conditions were evaluated using standard solutions of the expected monosaccharides. To be detected by the UV detector, the monosaccharides were subjected to pre-column derivatization with 3-methyl-1-phenyl-2-pyrazoline-5-one (PMP).

Different mobile phases consisting of a buffer and varying concentrations of acetonitrile (ACN) were tested. Ammonium acetate buffer at a concentration of 0.1 M and a pH of 5.5 together with ACN gradients over 30 min from 17 to 22 %, as employed by Stepan and Staudacher (2011),

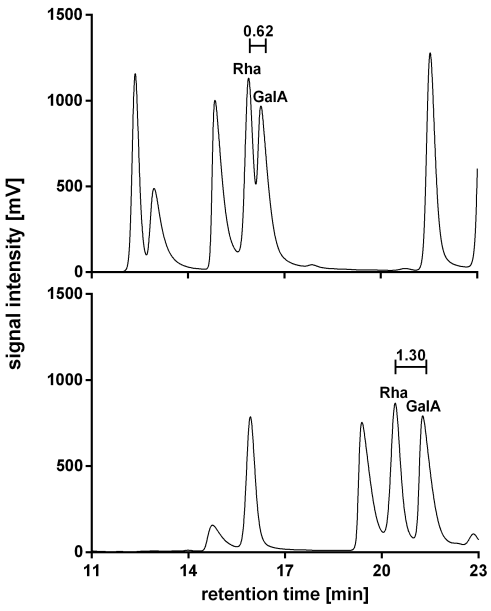


Figure 3.12: Resolution (indicated above the peaks) of rhamnose (Rha) and galacturonic acid (GalA) by C18 reversed phase-HPLC at two gradient conditions; top: increasing ACN concentration from 0 min bottom: 5 min isocratic then increasing ACN concentration

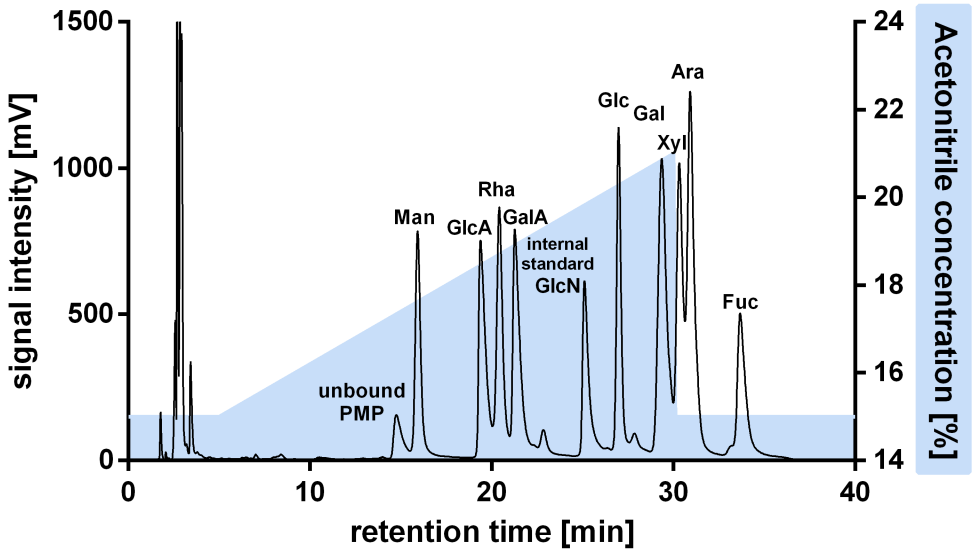


Figure 3.13: Monosaccharides labeled with PMP in individual concentrations and separated by reversed phase HPLC with 50 mM sodium phosphate buffer (pH=6.5) and ACN as mobile phase, the gradient of ACN concentration is shown in blue

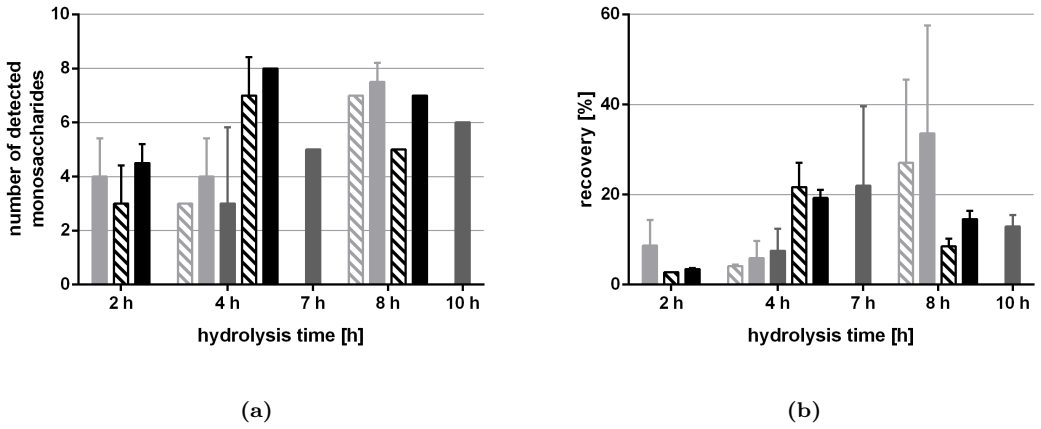
resulted in undesirable tailing of the peaks. The use of 50 mM sodium-phosphate buffer at pH 6.3 to 6.7 showed more promising results. Unfortunately, gradients applied by Yang et al. (2005) or previously tested isocratic conditions (Dai et al., 2010) either showed unsatisfactory resolution or inconveniently high retention times of the sugar derivatives.

A variation of the gradient applied by Yang et al. (2005) showed the most satisfactory results. An initial isocratic phase of 5 min followed by an increase in ACN concentration from 15 to 21 % resulted in slower overall elution compared to the same gradient starting at 0 min. Furthermore, the constant ACN concentration at the beginning doubled the resolution of the early eluting rhamnose and galacturonic acid (Fig 3.12).

Fig. 3.13 shows an exemplary chromatogram produced by the established gradient. The chromatogram shows all used standards including the internal standard glucosamine, which is not found in the analyzed algal polysaccharides.

Enhancing Hydrolysis Output

Hydrolysis of polysaccharides is a necessary but also critical step in the analysis of their monosaccharide composition as suboptimal conditions diminish the yield or may destroy certain components. Optimal conditions vary for each



(a) (b)

Figure 3.14: Yield after various hydrolysis conditions in number of detected monosaccharides (a) and total recovery in percentage of applied dry weight (b) in dependence of time [h], TFA concentration [M] and temperature [℃], n=2

polysaccharide and therefore have to be determined individually. In order to find a satisfactory temperature, duration, and acid concentration, hydrolysis at various conditions was performed on one batch of EPS, isolated from *Netrium digitus*.

Only one of the tested conditions resulted in the detection of eight monosaccharides (Fig. 3.14a). Low temperatures (80 and 95 ℃) combined with a short time (2 h) only hydrolyzed three to five monosaccharides and, in addition, showed a large standard deviation in total yield (Fig. 3.14b). While the number of detected monosaccharides and total recovery increased with time at 80 ℃, at 110 ℃ and 8 h a decrease in total recovery compared to 4 h was observed. Also, the number of detected monosaccharides was lower. The intermediate temperature of 95 ℃ showed results between the other temperatures. The highest number of monosaccharides was detected at 10 h and 95 ℃, but total yield was maximal at 7 h. In general, a higher TFA concentration produced higher numbers of monosaccharides and total yields compared to the lower concentration under otherwise comparable conditions.

Yields of the single monosaccharides of the applied EPS for the most favorable conditions are shown in Fig. 3.15. Xylose and an unknown component decreased considerably with increasing temperature and duration. Similar effects, but to a lesser extent, were observed for galactose, arabinose, and fucose. On the other hand, more glucuronic acid was hydrolyzed under harsher conditions. Rhamnose and glucose showed the least variability under the indicated conditions.

Due to the considerably smaller standard deviation in total yield and high

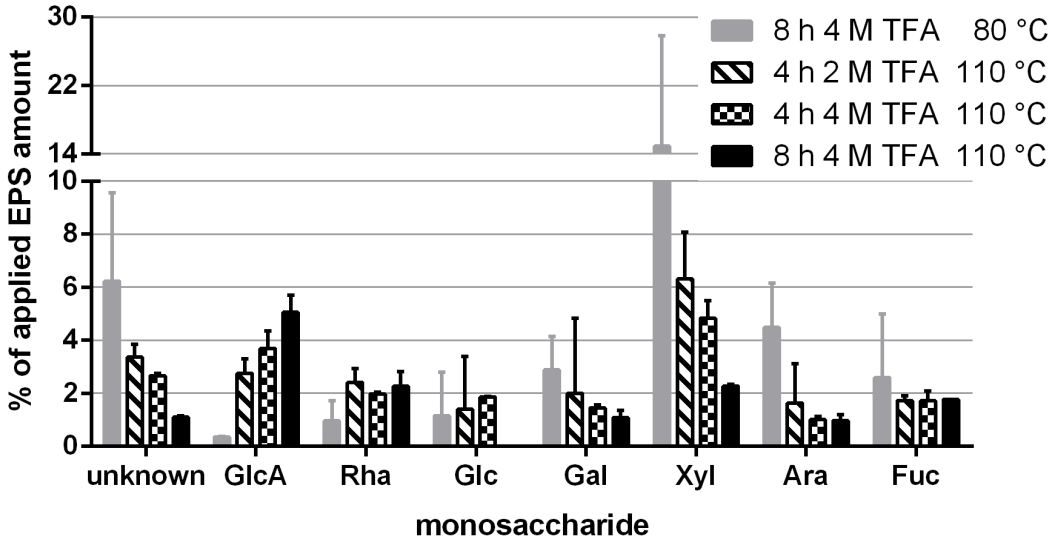


Figure 3.15: Percentage yield of individual monosaccharides per unit of applied EPS for selected hydrolysis conditions, n=2

number of detected monosaccharides, 110 °C for 4 h and with 4 M TFA were chosen for further routine hydrolysis. The total yield was further improved from 19.23 ± 1.27 % to 55.16 ± 10.71 % (average of yields of all TL experiments) by decreasing the temperature at the evaporation step after hydrolysis.

EPS Purification

Cleaning the fraction of interest in a sample always is a trade-off of yield and purity. After isolating EPS from *Netrium* cells by centrifugation, two purification steps were compared to no treatment. The combined recovery of monosaccharides of the applied dry weight was with 36.66 ± 5.75 % lowest for the non-purified samples. Precipitation (45.73 ± 3.62 %) and dialysis (49.62 ± 4.87 %) yielded more, but the differences were not significant.

The composition as shown in Fig. 3.16 only varies significantly for glucose between the non-treated and the dialyzed samples, with a higher percentage in the non-purified samples.

The combination of precipitation and dialysis was not investigated in this evaluation, but in the following growth experiments (suspension and TL) EPS purification was achieved by first precipitating the polysaccharides, drying the samples, determining the dry weight and then dialyzing the resuspended samples. After the previously described optimizations had been evaluated, a further modification was introduced. The temperature for evaporation of TFA after

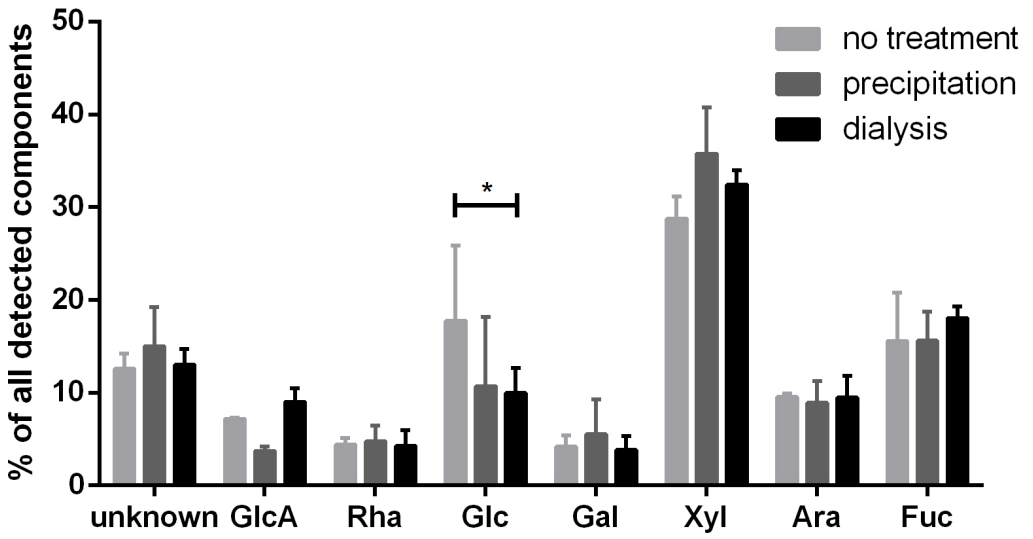


Figure 3.16: Monosaccharide composition of *Netrium digitus* EPS in percent of the analyzed fraction after different purification steps; * p-value ≤ 0.05 ;n=3

hydrolysis was reduced (35 ℃) for all growth experiment samples. The there obtained monosaccharide compositions are therefore different from the previously presented.

3.3.2 Composition of *Netrium digitus*' EPS in Suspension

The monosaccharide composition of EPS was analyzed at two time points of the suspension culture previously described (Sec. 3.1, p. 31). The first sample was taken on day 12 in the late exponential growth phase. A second sample from the stationary phase (day 18) was also analyzed. The results are shown in Fig. 3.17. Ten different monosaccharides were detected. Compared to the previously presented compositions in the methodology section, mannose and galacturonic acid were additionally detected. Mannose though was only recovered in the older sample with a low concentration of 0.8 %. The galacturonic acid content of 0.23 and 0.34 % was even lower. Rhamnose, glucose, and galactose constituted about 3 %, each. The only significant difference between the samples was the arabinose content. In the early sample, it was 7.24 % and decreased to 4.49 % in the EPS harvested on day 18. Glucuronic acid was present at a quantity of 14.21 %, which is similar to the unknown component with 13.55 %. Both values are an average of the two time points. The two most abundant components were fucose (17.25 %) and by far xylose (39.36 %).

In all EPS samples from *N. digitus* one unknown component, eluting between

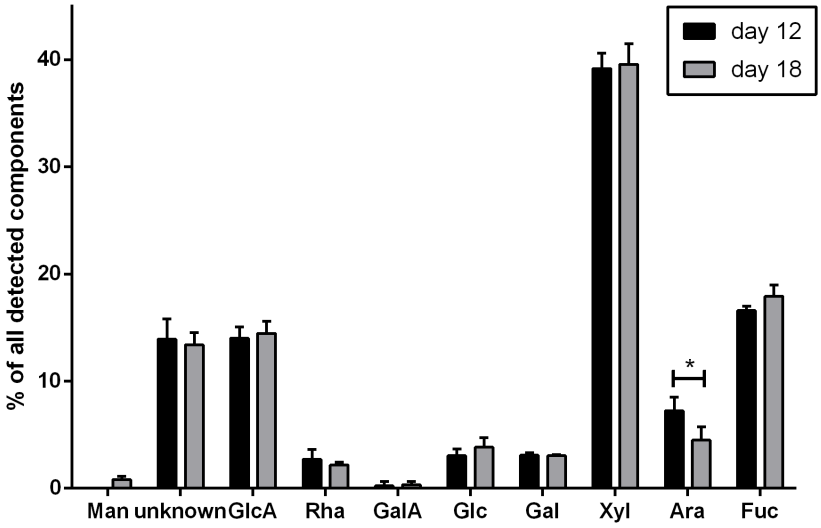


Figure 3.17: Monosaccharide composition of *Netrium digitus* EPS in percent of the analyzed fraction at two time points during the suspension culture, * p-value ≤ 0.05; n=3

mannose and glucuronic acid, was detected. The considerable peak area called for further investigations. The possibility that this component is only an artifact from hydrolysis or derivatization was excluded because these steps were also run with clean standards, which did not show a peak at around 16.5 min. As precipitation and dialysis could not be performed with monosaccharide standards, samples of *N. digitus* EPS were also analyzed after omitting these treatments one-by-one. In all cases, the component was detected.
In order to have quantitative results, the unknown was quantified using the response of the xylose calibrations.
Apart from polysaccharides, other molecules can be present in the EPS. A protein assay performed with culture supernatant samples, as well as concentrated EPS (5 mg·mL^{-1}), did not detect any protein. Since the detection limit of this assay was 0.5 mg·mL^{-1}, the possible protein fraction would be, if at all present, less than 10 %.

3.3.3 Composition of *Netrium digitus*' EPS on Twin-Layer

Extracellular polysaccharides, isolated from biofilm samples taken during TL cultivation, were also analyzed for their monosaccharide composition. Nine to ten different components were detected in each sample (Fig. 3.18). Galacturonic

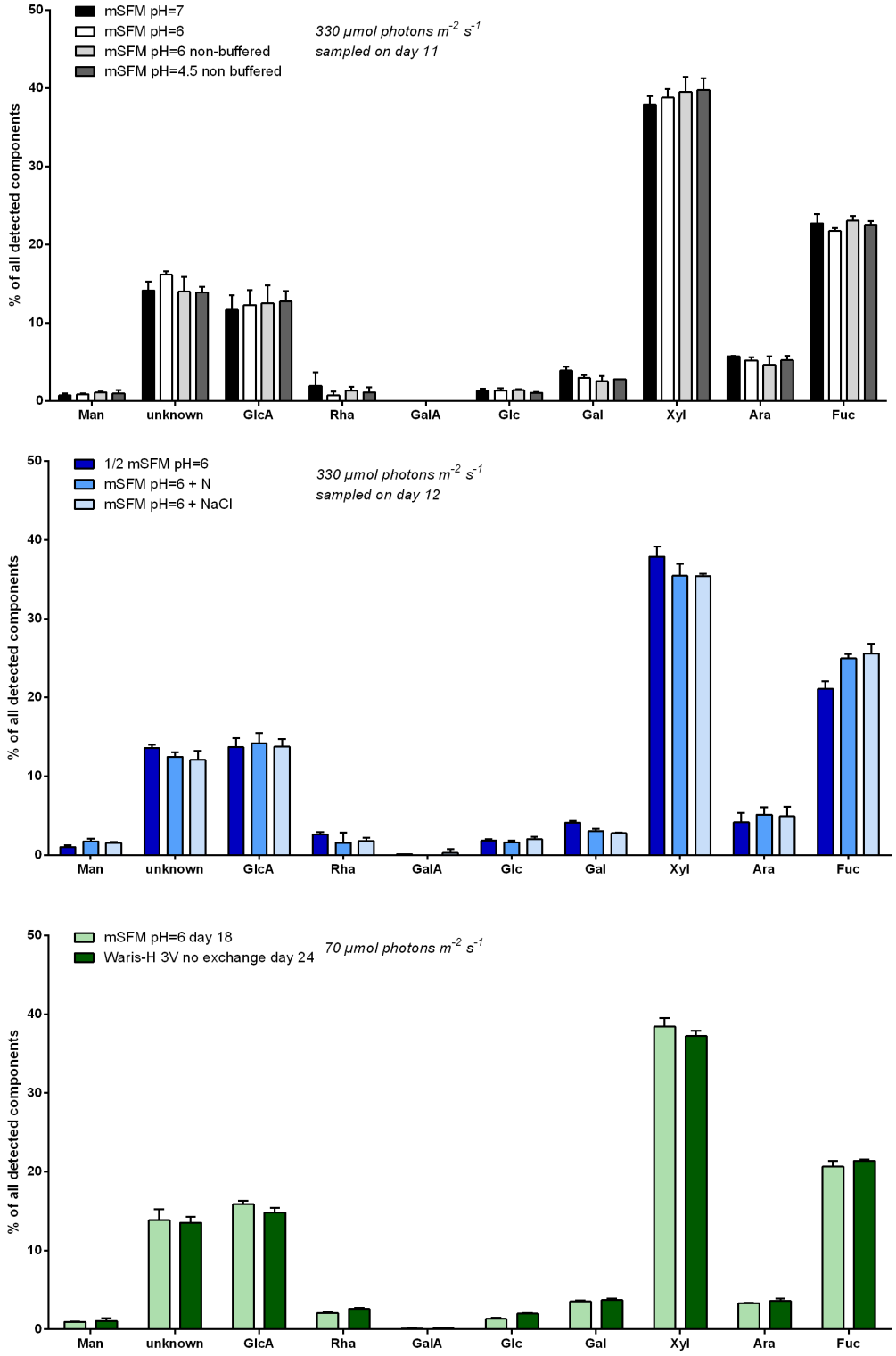


Figure 3.18: Monosaccharide composition of *N. digitus* EPS in percent of the analyzed fraction of nine TL cultivations, light intensity for cultivation and time of EPS samples are indicated in the legend, n=3

acid was not detected in all samples. The quantities were below 0.3 % in the four samples, in which GalA was detected at all. As the peaks were also very small, GalA was not detectable in the other samples, because it was below the detection limit. Rhamose was also only found in low concentrations. Between 0.74 % (mSFM pH=6) and 1.36 % (Waris-H no change) were detected. In contrast to the suspension culture samples, mannose was detected in all TL samples. The average mannose content in TL samples was around 1.15 %. Glucose and galactose were present in concentrations around 1.68 and 3.31 %, respectively. Higher concentrations were measured for arabinose (4.72 %), the unknown component (13.45 %) and glucuronic acid (13.36 %). The most abundant monosaccharides were fucose and xylose with 23.02 and 37.41 %, respectively.

Some differences between the suspension culture and the TL EPS were observed. Glucose was 51 % less abundant in EPS on TL than in suspension culture EPS. As already observed during the analysis of the suspension culture alone, more arabinose was found in the early suspension culture sample. Also, the TL samples contain on average 34.7 % less arabinose than this early sample. No difference in comparison to the second suspension culture sample was detected.

Xylose and fucose content also differed between suspension culture and TL cultivation. In all TL samples, fucose content was above 20 %. Compared to the average of the suspension culture (17.25 %) the average increase amounts to 33.4 %. The xylose content behaves differently. On average, 5 % more xylose is found in suspension culture EPS than in the TL EPS.

Comparison of the composition at different light intensities reveals that only 87.7 % of the glucuronic acid content in samples grown at low light were found in samples grown at high light. This includes the suspension culture.

3.4 Interaction of Desmids and Bacteria

3.4.1 Bacterial strains in *Cosmarium* Cultures

Xenic *Cosmarium humile* and *Cosmarium pachydermum* stationary phase cultures were plated on Waris-H 1/2 BSM with 1.5 % agar, and after four days the developed colonies were counted to determine the bacteria/alga ratio and to isolate single colonies for the growth experiments and rRNA gene sequencing.
Brief descriptions of the colony morphology together with the sequencing results are listed in Tab. 3.1. Based on the 16S rRNA gene sequences five different strains were isolated from one *C. humile* culture. However, by colony morphology six strains were distinguishable. The strains 1 and 2 both had an identical partial 16S rRNA gene sequence, indicating the affiliation to the *Cytophaga* genus. The strain forming red colonies was an *Arcicella aquatica* strain also belonging to the Bacteroidetes phylum. With *Asticcacaulis excentricus* (strain 4) a representative of the α-proteobacteria was identified. This strain showed the highest abundance of 10 bacterial cells per algal cell compared to the other five strains. Strain number 5 was identified as *Curvibacter gracilis*. An interesting result was the sequencing of strain 6. The obtained sequence showed 16 differences and two gaps compared to the closest match, which, in addition, is part of a group of only uncultured bacteria isolated from platinum mine material (NCBI Information on accession nr. JN030513). The closest related, identified bacteria belonged to the genus *Rhizobium* and *Neorhizobium*.
Only one strain was isolated from the xenic *C. pachydermum* culture. The ratio of algae to this *Pseudomonas moraviensis* was 1:184. This is much higher than all combined bacterial cells per alga in the *C. humile* culture. It has to be emphasized, that *C. pachydermum* has an approx. 190 times higher cell volume than *C. humile*.

3.4.2 Growth in Xenic and Axenic Algal Cultures

The growth of the zygnematophyceaen algae *Cosmarium humile*, *Cosmarium pachydermum* and *Netrium digitus* under xenic and axenic conditions was monitored by cell counts. In the cases of *C. humile* and *C. pachydermum*, axenic cultures were re-inoculated with bacteria previously isolated from the corresponding xenic culture (cf. previous section) in order to see the effect of a single bacterial strain.
Algal growth in all seven *C. humile* cultures, presented in Fig. 3.19, followed the same pattern. Starting from the initially equal cell density of 341·mL^{-1} the

Table 3.1: Bacterial colonies isolated from xenic *C. humile* and *C. pachydermum* cultures with colony description, average number per alga in stationary phase of alga, results of nBLAST (2016-04-16) together with sequence similarity [base changes/sequence length]

associated alga	internal no.	colony description	bacteria/ alga	closest relative title accession number	base changes/ sequence length
C. humile	1	yellow-orange	3	Uncultured bacterium clone JdFBHulkDF73 *Cytophaga* sp. JQ678555.1	1/1375
	2	yellow	2	Uncultured bacterium clone JdFBHulkDF73 JQ678555.1	1/1375
	3	white (brown in the light)	10	*Asticcacaulis excentricus* strain CB 48 NR_074137.1	1/1253
	4	red	4	*Arcicella aquatica* strain NO-502 NR_029000.1	1/1256
	5	white with fuzzy edges	1	*Curvibacter gracilis* strain 7-1 NR_028655.1	5/1370
	6	white, transparent	1	Uncultured bacterium clone NO24ab16S15 *Rhizobium* sp. JN030513.1	16/1337 and 2 gaps
C. pachydermum	7	white, irregular colony shape, motile	184	*Pseudomonas moraviensis* strain IARI-HHS1-33 KF054775.1	0/1364

cell number was doubled within 1.60 ± 0.02 days for cultures containing bacteria and within 1.68 ± 0.01 days for the axenic culture. These exponential phases lasted until day 14, after which cell division slowed down. The final cell numbers ranged from $1.90 \pm 0.25 \cdot 10^5$ for *C. humile* co-cultured with *Cytophaga* sp. 1 to $3.97 \pm 0.88 \cdot 10^5$ for the culture containing *Curvibacter gracilis*. Significant differences in the final cell numbers were only detectable between the *Cytophaga* containing cultures and the *Curvibacter gracilis* culture.

Even though the differences in cell number at the end of the experiment were not pronounced, the calculated growth rate between day 1 and 14 was significantly

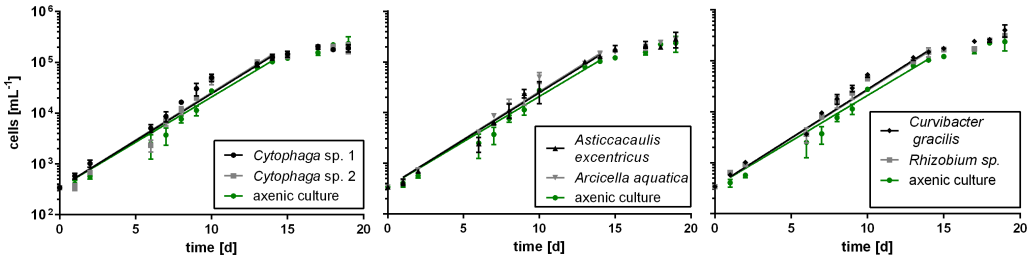


Figure 3.19: Growth of *Cosmarium humile* in axenic state and with addition of one bacterial strain as indicated, inoculation in the bacteria/alga ratio as stated in Tab. 3.1, n=3

lower for the axenic culture compared to every of the bacteria-containing ones. The bacterial growth was roughly monitored by plating out dilutions. Over the course of the growth experiment, their numbers increased until around day 15, when the algae growth also ceased. Apart from the desired bacterial colonies no contaminations were visible on the plates.

Analogous to the growth experiment with *C. humile*, *C. pachydermum* was co-cultured with the previously recovered *Pseudomonas* sp. strain (Fig. 3.20). The much larger algal cells than *C. humile* showed a shorter exponential phase, only lasting until day 10, with also higher doubling times of 2.48 ± 0.05 days and 2.65 ± 0.06 days for the xenic and the axenic culture, respectively. The axenic *C. pachydermum* culture also showed a significantly lower growth rate than the co-culture. The final cell numbers of $1.88 \pm 0.75 \cdot 10^4$ (xenic) and $1.37 \pm 0.13 \cdot 10^4$ (axenic) again did not differ significantly.

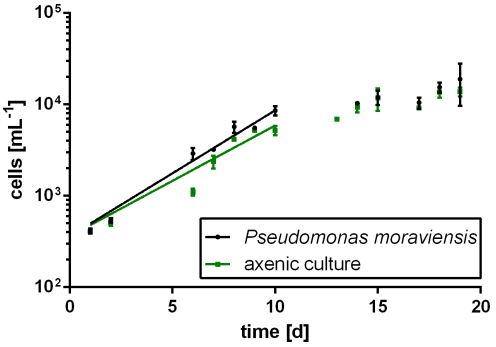


Figure 3.20: Growth of *Cosmarium pachydermum* in axenic state and with addition of *Pseudomonas* sp. in an alga:bacteria ratio of 1:184, n=3

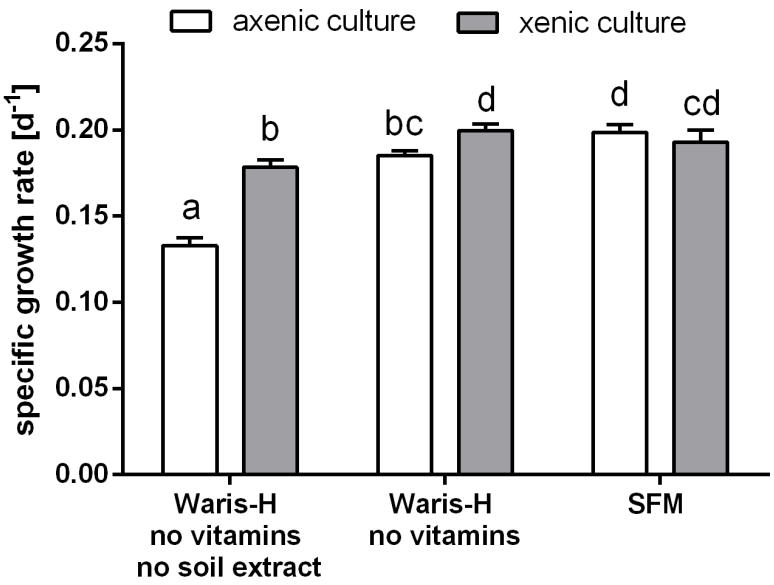


Figure 3.21: Specific growth rates of *N. digitus* calculated after 12 days in indicated growth medium as axenic or xenic culture with comparable starting cell density, letters indicate significant differences (p-value $\leq$0.05) based on two-way ANOVA, n=3

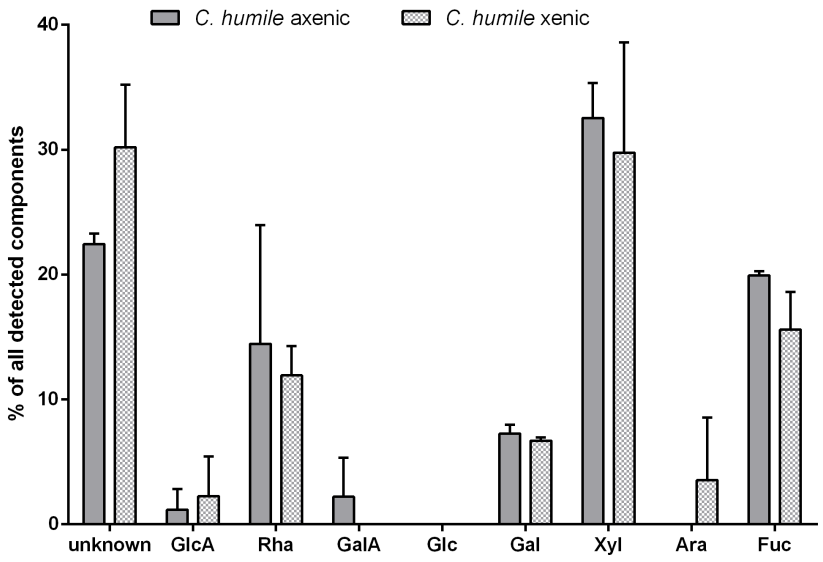
Specific growth rates of *Netrium digitus* in different growth media and under axenic and xenic conditions were determined and are shown in Fig. 3.21. As the letters indicate, growth of the axenic culture in Waris-H without vitamins nor soil extract was with 0.13 d^{-1} significantly lower than in all the other cultures. The xenic culture in the same medium reached a growth rate of 0.18 d^{-1}. Both cultures in Waris-H with soil extract but without vitamins, as well as the cultures in SFM, showed similar values between 0.19 d^{-1} and 0.20 d^{-1}. The final cell number of *N. digitus* in Waris-H without soil extract and vitamins was only $4.09 \pm 0.68 \cdot 10^3$ cells per milliliter and thus significantly lower than in the other cultures. The xenic culture in this medium reached $9.47 \pm 0.96 \cdot 10^3 \cdot mL^{-1}$, which also is significantly less than the final cell number in axenic Waris-H without vitamins ($1.43 \pm 0.14 \cdot 10^4 \cdot mL^{-1}$) and in the axenic SFM culture ($1.35 \pm 0.18 \cdot 10^4 \cdot mL^{-1}$). Intermediate values were counted for the xenic cultures in Waris-H without vitamins and SFM with $1.09 \pm 0.86 \cdot 10^4 \cdot mL^{-1}$ and $1.22 \pm 0.37 \cdot 10^4 \cdot mL^{-1}$, respectively.

3.4.3 EPS Concentration and Composition in Xenic and Axenic Cultures

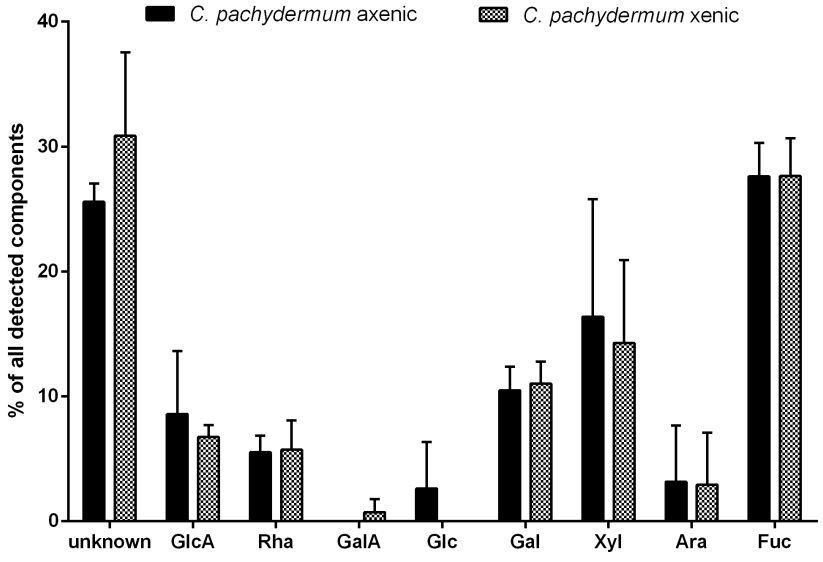
For the compositional analysis of polysaccharides produced by axenic and xenic cultures (not the addition of isolated bacterial strains), the EPS were isolated under comparable conditions and therefore an estimation of the EPS production can be made. The axenic *Cosmarium humile* culture ($3.4{\cdot}10^5$ cells per mL) contained 787.5 mg·L^{-1} of EPS. The xenic culture ($5.4{\cdot}10^5$ cells per mL) only contained 138.8 mg·L^{-1}. For *Cosmarium pachydermum* the same trend was observed. The EPS concentration of the axenic culture ($6.9{\cdot}10^3$ cells per mL) was measured to be 1180 mg·L^{-1} while the xenic culture ($6.9{\cdot}10^3$ cells per mL) only produced 508.1 mg·L^{-1}. In contrast to these measured values, the observed viscosity was higher in both xenic cultures compared to the axenic ones. By measuring the time a metal bead needed to fall through a solution of these isolated polysaccharides of equal concentration this optical observation was confirmed.

The results of the monosaccharide composition are presented in Fig. 3.22. For *Cosmarium humile* (Fig. 3.22a) the most prominent monosaccharide was xylose with 32.54 ± 1.98 % in the axenic sample and 29.77 ± 6.24 % in the xenic. The unknown component had the same retention time and is most likely the same as the one found in *Netrium* EPS (cf. Sec. 3.3.2, p. 48) and constituted for 22.43 ± 0.60 % and 30.30 ± 3.53 % for axenic and xenic EPS, respectively. This difference between axenic and xenic culture was significant. The next most abundant monosaccharides were fucose, rhamnose, and galactose. Arabinose and galacturonic acid were only detected in one of the four samples. Glucuronic acid was only detected in one of the two replicates of each culture type. It can be concluded that these monosaccharides only occurred in quantities close the detection limit and therefore no quantification was possible.

The EPS composition of *Cosmarium pachydermum* cultures showed a different pattern (Fig. 3.22b). Here, the most abundant monosaccharide was fucose with 27.62 ± 1.89 % and 27.64 ± 2.15 % for axenic and xenic samples, respectively. The unknown component again was very prominent with 25.58 ± 1.03 % and 30.87 ± 4.73 %. Third most was xylose with 16.39 ± 1.34 % and 14.28 ± 4.68 %. Galactose was with 10.49 ± 1.34 % and 11.03 ± 1.24 % more abundant than in *Cosmarium humile* or *Netrium digitus* EPS. Glucuronic acid, rhamnose, and arabinose were present in small quantities. Glucose and galacturonic acid were only found in one of the four samples and quantification was not possible.



(a)



(b)

Figure 3.22: Monosaccharide composition of EPS produced by *Cosmarium humile* (a) and *Cosmarium pachydermum* (b) cultures in axenic and xenic state, n=2

3.4.4 Antibiotic Properties of Algal and Bacterial Cultures

Plate diffusion tests with algal cultures and their corresponding bacterial cultures were performed in order to reveal possible antibiotic properties against *Bacillus subtilis* and *Klebsiella trevisanii*. Xenic cultures of *Netrium digitus* (M3119) and *Cosmarium subprotumidum* (M3049) showed inhibition zones for *Bacillus subtilis*.

Bacteria isolated from these cultures were also tested and showed the same effect. Samples of axenic algal cultures though did not show any inhibition of bacterial growth. The antibiotic effects can be attributed to the bacteria and were quantified to be equivalent to 240.61 µg·mL^{-1} and 205.60 µg·mL^{-1} of kanamycin for bacteria isolated from *N. digitus* and *C. subprotumidum* cultures, respectively.

3.5 EPS Quantification with Ruthenium Red

An easy and rapid spectrophotometric method for EPS quantification was developed using Ruthenium Red (RR). Acidic polysaccharides precipitate together with RR and result in a decrease in color of the remaining solution. The negative linear correlation of polysaccharide amount and absorbance of supernatant can conveniently be expressed as absorbance difference to a sample not containing any polysaccharides in dependence of original polysaccharide concentration. A good correlation between bound RR and EPS dry weight during the course of a suspension culture of *Netrium digitus* is shown in Fig. 3.23. A low RR concentration of 12.7 mg·L^{-1} showed strong linearity in an assay with xanthan for volumes of 1 mL and cuvettes for measurement (Fig. 3.24). Comparable results were also obtained for EPS from Zygnematophyceae.
Down-scaling to microtiter format (200 µL) decreased the slope because the path length of the light is much shorter (Fig. 3.24). The use of microtiter plates for spectrophotometric methods poses great advantages and therefore efforts were made to increase the slopes. Variations of RR concentration and pH of the

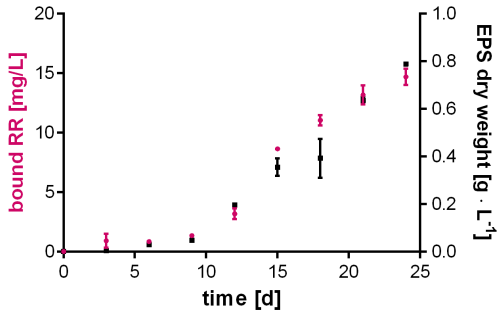


Figure 3.23: Bound RR after reaction with culture supernatant samples of *Netrium digitus* and EPS dry weight measured by gravimetry over 24 days using 12.7 mg·L^{-1} RR and no pH adjustment, n=3

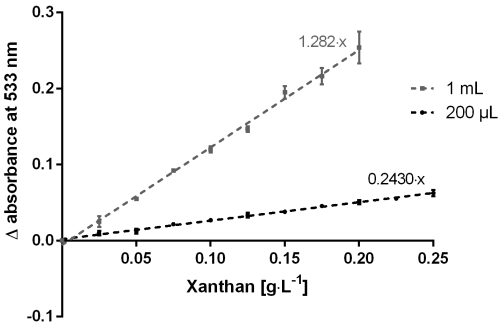


Figure 3.24: Standard curve with xanthan in RR assay in 1 mL and 200 µL scale, difference in absorbance to polysaccharide-free sample, RR concentration in reagent solution 12.7 mg·L^{-1} no pH adjustment, n=3

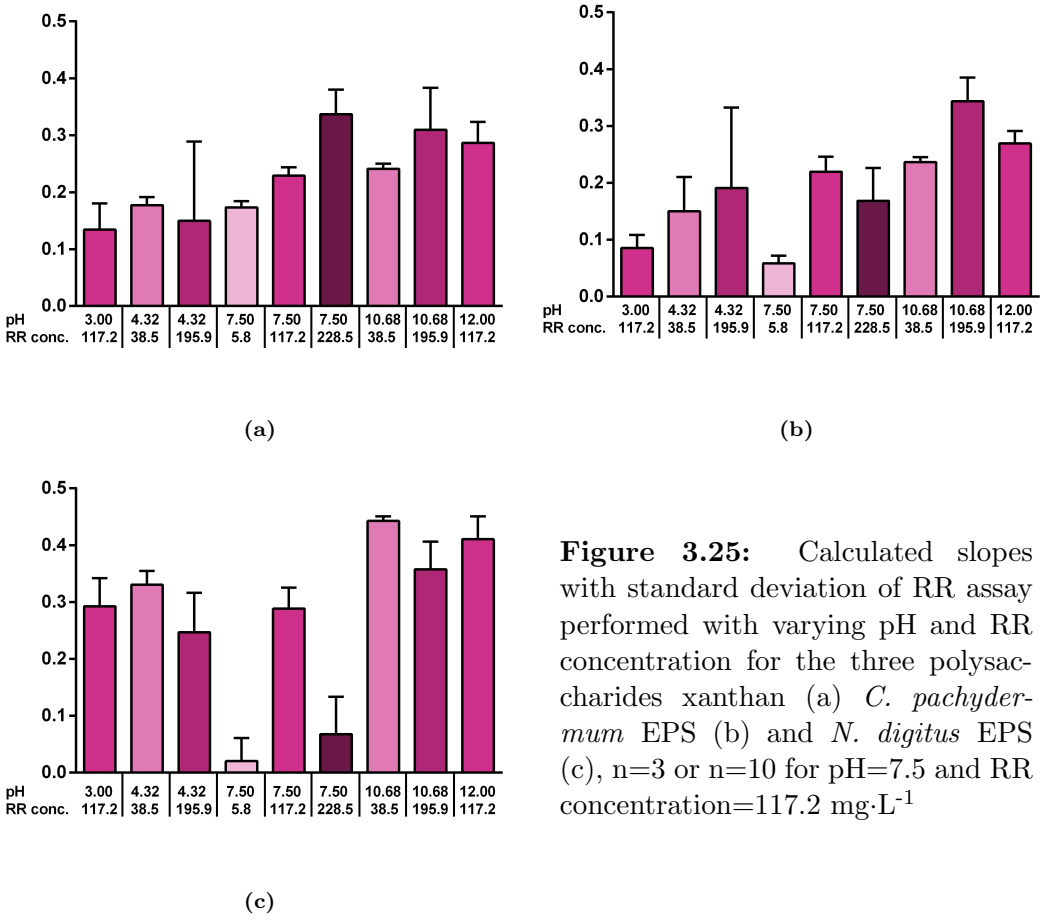


Figure 3.25: Calculated slopes with standard deviation of RR assay performed with varying pH and RR concentration for the three polysaccharides xanthan (a) *C. pachydermum* EPS (b) and *N. digitus* EPS (c), n=3 or n=10 for pH=7.5 and RR concentration=117.2 mg·L^{-1}

reagent solution were tested using a central composite design as experimental design. This systematic approach allowed the screening of the broad range of experimental conditions listed in Tab. 2.9 (p. 28) for xanthan as well as EPS isolated from *Netrium digitus* and *Cosmarium pachydermum* cultures. The slopes were calculated over the whole range of EPS concentrations (0-300 mg·L^{-1}) apart from the lowest RR concentration 5.93 mg·L^{-1}, which is not sufficient to precipitate EPS of higher concentrations than 0.15 g·L^{-1}. In this case, the linear range was limited between 0 and 0.15 g·L^{-1} and slopes were only calculated within this range.

The slopes of the nine conditions for the three polysaccharides are shown in Fig. 3.25. The shallowest slope was measured using 5.83 mg·L^{-1} at almost neutral pH and *N. digitus* EPS with only 0.020. The highest slope of 0.443 was also measured with this EPS, namely with 38.53 mg·L^{-1} RR and pH=10.68. Using xanthan as a standard polysaccharide resulted in slopes between 0.134 and 0.337 for 117.23 mg·L^{-1} with pH=3.00 and 228.53 mg·L^{-1} with pH=7.5, respectively. As for *Netrium* EPS, *Cosmarium pachydermum* EPS also showed only little change in absorbance when 5.833 mg·L^{-1} RR with pH=7.5 were used

(slope=0.058). The maximal slope for this polysaccharide was 0.344 under the conditions of 195.93 mg·L^{-1} and pH=10.68. In general, it can be said that a high pH increased the slopes. The influence of the RR concentration was not so clear and showed mixed tendencies for example at pH=7.5. For xanthan the trend is clear: the higher the concentration, the higher was the slope. For the two zygnematophyceaen EPS the maximal slopes at this pH were at 117.2 mg·L^{-1}. The figures also depict the standard deviation. Especially pH=4.32 and 195.9 mg·L^{-1} RR showed large variation with all three polysaccharides. The lowest standard deviation was found at pH=10.68 and 38.5 mg·L^{-1}.

A quantitative assay can only be reliable when the linear range is satisfactory and the variability of the reaction is minimal. In all conditions, apart from the aforementioned low RR concentration of 5.93 mg·L^{-1}, the absorbance difference was linear up to the maximally measured EPS concentration of 0.3 g·L^{-1}. The application range of the assay is also limited by the viscosity of the samples. EPS concentrations of 0.3 g·L^{-1} and higher become increasingly viscous which may also lead to pipetting errors, especially at such low volumes of 20 µL, as used in the presented assay. Taking the limited linearity of the assay and the viscosity into consideration a maximal EPS concentration of 0.3 g·L^{-1} is recommended and can also be easily estimated by means of the viscosity as the solutions show a delay in being pulled into the pipette tip around this concentration. Direct comparison of the standard curves at the starting condition (pH=7.00 and 12.7 mg·L^{-1} RR) with the best condition of the screening (pH=10.68 and 38.5 mg·L^{-1} RR) in Fig. 3.26 shows a clear increase for the zygnematophyceaen EPS but not for xanthan. While the slope for *N. digitus* EPS was 3.6-fold and for M2872 EPS 1.9-fold increased, the slopes for xanthan did not differ significantly.

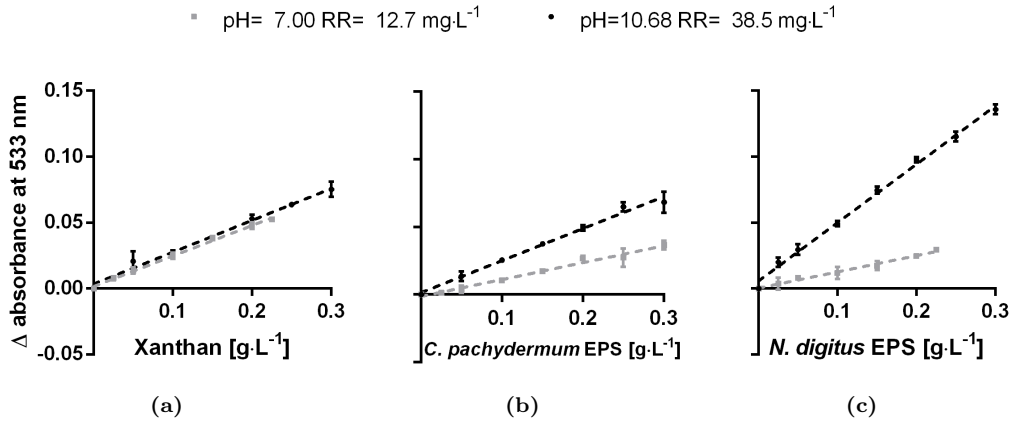


Figure 3.26: Comparison of slopes of xanthan (a), *C. pachydermum* EPS (b) and *N. digitus* EPS (c) at pH=7.00 and 12.7 mg RR·L^{-1} and at pH=10.68 and 38.5 mg RR·L^{-1} with standard deviation, n=3

In all cases, the higher pH and RR concentration show linearity and can be used for EPS quantification.
During the work on this RR assay, some additional issues concerning the assay were observed. The stability of RR at room temperature and possibly light is limited. Measurable decreases in absorbance were observed after 24 h at room temperature. On the other hand, storage at -20 ℃ prevented degradation.
Mixing is of particular importance due to the partially high viscosity of the samples and the accompanied slow mixing by diffusion. Assay performance in reaction tubes and vortexing showed good results, but thorough mixing by repeated pipetting is also possible. In this case, precipitated RR-EPS complexes may stick to the pipette tips, but as they are anyway removed by centrifugation and transferal of the supernatant this does not cause a problem.
Time for incubation prior to centrifugation was tested and showed increased variability in the absorbance measurements. It is therefore recommended to proceed with the assay without any delay.

4. Discussion

4.1 Growth and EPS Production of *Netrium digitus* in Suspension

The preliminary media test with non-aerated cultures shed some light on the media requirements of *N. digitus*. The highest growth rate was achieved in SFM, before Waris-H without vitamins and Waris-H without vitamins nor soil extract. These results clarify two things. First, the addition of soil extract to Waris-H is clearly necessary for good growth of axenic *Netrium digitus* (the influence of bacteria is discussed in Sec. 4.5, pp. 87). The nutrient analysis also emphasizes this by showing that nutrient uptake was reduced and the lower growth rates, therefore, do not result from nutrient depletion. Secondly, even though SFM does not contain any soil extract, the cells showed good growth. Therefore, the medium must contain the element, which is lacking in the Waris-H without soil extract. Comparison of the media compositions reveals six possible candidates, viz. vanadium, selenium, nickel, molybdenum, copper and chromium, which are present in SFM, but not in the salt stock solutions of Waris-H.
The yellowish color and the unhealthy looking chloroplasts in SFM grown cells indicate nitrogen limitation, which is confirmed by the nutrient analysis. Chlorosis is an early sign for this circumstance (LaRoche et al., 1993). The reasons why the cells in Waris-H without vitamins do not present the symptoms of nitrogen limitations are that on day 14 still 14.93 % of the initial nitrate were available and that Waris-H contains ammonium as a second nitrogen source.

A more resolved growth curve together with EPS concentration measurements was performed using Waris-H 3V as growth medium and supplying carbon dioxide to the culture. *Netrium digitus* was grown in Erlenmeyer flasks and cell number as well as EPS concentration were measured every three days. While the cells number increased exponentially until day 12, the EPS accumulated over the whole course of the experiment and even showed an increase in accumulation

when growth had already ceased.

The exponential growth is typical for suspension cultures. The doubling time of 2.37 days compares well with other zygnematophyceaen cultures as *Cosmarium abbreviatum* var. *planctonicum* and *Staurastrum chaetoceras* at comparable light intensities with 2.86 d and 2.00 d, respectively (Coesel and Wardenaar, 1994), even though the investigated cells were much smaller than *Netrium* and thus higher growth rates can be expected. The reason may lay in the carbon dioxide addition. *Cosmarium* and *Staurastrum* were only bubbled with humidified air, while *Netrium* was supplied with 1.5 % CO_2.

The cell dry weight per cell was calculated based on cell counts and dry weight determinations of two suspension cultures and biomass samples harvested from a TL-system (Waris-H 3V, 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$, day 21). The determination showed large differences between the samples, which are caused by the cell size variability that occurs with *Netrium* during the cell cycle and due to accumulation of storage compounds. The values, therefore, can only give an estimate rather than an exact value.

It can be concluded that with increasing culturing time the nutrients in the medium were depleted and, because no exchange was performed (in contrast to cultivation on TL), became limiting. In comparison to other algae than the Zygnematophyceae, the reached EPS concentration of 0.79 $g \cdot L^{-1}$ is high (cf. Tab. 4.1). Only *Porphyridium* sp. produced a comparable amount in only 16 days (Adda et al., 1986). The final concentration in one culture of *Botryococcus braunii* is also in the same range, but it took twice as long to be produced (Bayona and Garcés, 2014).

A comparison can be also made with other zygnematophyceaen algae (Tab. 4.2) and comparable harvesting and EPS quantification methods make the comparison more reliable. Since Domozych et al. (1993) did not state total EPS concentrations nor final cell numbers, comparing the EPS amount per cell is a reasonable approach. Differences of at least two orders of magnitude can be seen. These large differences are not diminished by calculating the EPS amount per cell volume. *Netrium* showed around 10-fold higher cell numbers after slightly shorter culture duration than *P.margaritaceum*, which equals out the lower EPS amount per cell and results in comparable final EPS concentrations under non-stressed conditions.

A condition, which is different between the here presented results and the literature values, and which also influences the EPS amount per cell, is the temperature. It was shown that lower temperature increased EPS production in *Netrium* (Kattner et al., 1977). The higher light intensity in Domozych's

cultures probably also led to a higher EPS amount (q.v. Sec. 4.3.2, p. 73) as well as the longer duration of cultivation. It is not clear if these differences in culturing conditions caused the grand discrepancy in EPS amount per cell or if *N. digitus* in general produces less mucilage than the investigated strains of *Closterium* sp. and *Penium margaritaceum*. What can be concluded from the data though is, that further increase of the EPS concentration can be achieved by altering the culturing conditions. The positive influence of e.g. high nitrogen on the EPS production was shown for *Penium margaritaceum*.

4.2 Comparison of Suspension Culture and Twin-Layer

4.2.1 General Differences Between the Systems

Immobilized microalgae cultivation is gaining popularity among cultivation techniques (Berner et al., 2015b). The high biomass concentration reduces energy and costs necessary for harvesting and dewatering the biomass in suspension culture. This main advantage of biofilm cultivation also leads to some fundamental different growth parameter compared to suspension culture. Combined with the fact that most, and in the case of Zygnematophyceae virtually all, of microalgal cultivation literature is based on work with suspension cultures difficulties comparing the results, obtained with two different systems, may arise.

First, the light situation is very different in both systems. By mixing or at least aerating a suspension culture all cells have a theoretical chance of being at the surface of the flask and therefore at the highest possible light intensity. The culture is thought to be homogeneous with random shading events and minimal deviation concerning the average light intensity, oxygen, and carbon dioxide concentration as well as pH.

Here, the first restriction has to be made for *Netrium digitus*. Because of the high viscosity of the suspension, mixing of the cells by aeration alone was not possible after day 15. Therefore, before every harvest (every 3 days), the suspensions were mixed on a magnetic stirrer. By this measure, a homogeneous culture was ensured.

Table 4.1: Comparison of EPS concentration in suspension culture of *Netrium digitus* to data found in literature

Species	***Netrium digitus***	***Chaetoceros affinis***	***Porphyridium UTEX 637***	***Chlamydomonas mexicana***	***Botryococcus braunii***
class	Zygnemato-phyceae	Bacillariophyceae	Rhodophyceae	Chlorophyceae	Trebouxiophyceae
light [μmol photons $\cdot m^{-2} \cdot s^{-1}$]	70	270	150	175	60
Temperature [$^\circ$C]	23	13	24	20	21
light:dark [hh:hh]	14:10	14:10	NA	16:08	24:00
Time [d]	24	12	16	24	49
pH	7	NA	NA	7	7.2
Medium	Waris-H 3V	f/10	artificial sea water	M3 medium	BG11 D medium
Remarks	+ CO_2	N:P 122:1	10 mM N and 2 % CO_2	aeration	+ CO_2
Harvest method	centrifugation, precipitation	filtration 20 µm	centrifugation	centrifugation	centrifugation, precipitation, dialysis
EPS quantification	weight	PSA	PSA	Anthrone method	PSA
EPS [g·L^{-1}]	0.79	0.04	0.75	0.54	0.47 0.65
cell density [cells·mL^{-1}]	$2.14 \cdot 10^4$	$2.0 \cdot 10^6$	$6.0 \cdot 10^7$	$7 \cdot 10^6$	0.9 g/L 0.75 g/L
EPS/cell [ng·cell^{-1}]	36.84	0.02	0.0125	0.077	0.52 g·g^{-1} 0.86 g·g^{-1}
Reference	this work	Myklestad and Haug (1972)	Adda et al. (1986)	Kroen (1984)	Bayona and Garcés (2014)

Table 4.2: Comparison of EPS concentration in suspension culture of *Netrium digitus* to data found in literature concerning the class of Zygnematophyceae

Species	***Netrium digitus***	***Closterium sp.***		***Penium margaritaceum***	
light [µmol photons ·m^{-2}·s^{-1}]	70	137	137	160	160
Temperature [°C]	23	17	17	18	18
light:dark [hh:hh]	14:10	14:10	14:10	14:10	14:10
Time [d]	24	30	60	28	28
pH	7	7.2	7.2	7.2	7.2
Medium	Waris-H 3V	Woods Hole Medium		Woods Hole Medium	
Remarks	+ CO_2	shaking		10 mM NH_4Cl	-
Harvest method	centrifugation, precipitation	centrifugation		centrifugation, precipitation, dialysis	
EPS quantification	weight	weight		weight	
cell volume [µm^3]	3.45·10^5	7.36·10^5		0.277·10^5	
EPS [g·L^{-1}]	0.79	NA	NA	1.75	0.75
cell density [cells·mL^{-1}]	21.4·10^3	NA	NA	0.70·10^3	1.75·10^3
EPS/cell [µg·cell^{-1}]	0.037	3.50	6.00	2.50	0.43
Reference	this work	Domozych et al. (1993)		Domozych (2007)	

When microalgae are grown in an immobilized state, the outermost cell layers shield the underlying cells from a majority of the light. Thus, a light gradient is established over the depth of the biofilm (Li et al., 2016). The cells on the surface are exposed to a high light intensity, while the cells of deeper layers only receive little light. Due to the immobilization, all cells are able to adapt to their light situation e.g. by pigment accumulation (Remias et al., 2012) and possibly orientation towards the light source (Nossag and Kasprik, 1993). Close observation of biofilms grown at 70 µmol photons ·m^{-2}·s^{-1} showed that the majority of the cells were orientated with their long side exposed to the light. This indicates that this light intensity is not stressful for the alga because otherwise the cells would have rotated into an apical orientation and thereby would have reduced the exposure to the light. The EPS secretion might even be used to move towards the light source, as the shaded positions inside the biofilm were not as favored as the surface positions. At the high light intensity, more cells on the surface were found in the apical orientation towards the light as well as more cells in the lower layers of the biofilm. These observations were not statistically quantified, yet.

Not only light but also gasses are available in their specific gradient over the depth of the biofilm (Li et al., 2016). The produced oxygen accumulates in the photosynthetically most active regions but exchange with the surrounding atmosphere is also facilitated compared to a suspension culture, where the gas molecules have to diffuse through several centimeters of water. The gradient of CO_2 is established vice versa.
In the TL system, carbon dioxide was applied to the gas phase and in the suspension culture it was bubbled into the liquid. In both cases, not all CO_2 may have reached the cells. Nevertheless, by applying the same CO_2 concentration at a continuous flow over the whole experimental period the cells should have never been CO_2 limited.
The higher cell density on the surface of the biofilm may not only be caused by the light gradient but also by the gradient of CO_2. Entrapped gas bubbles in the biofilms indicate that diffusion is hindered, which limits the growth in thicker biofilms because carbon dioxide is not diffusing sufficiently into the inner regions.
Another important difference between the two systems is their interaction with the surrounding environment. Suspension cultures can be maintained in an axenic state as long as there is a barrier for other organisms (e.g. bacteria, fungi and other microalgae) and no contamination takes place during harvest and inoculation. The TL system, at least the set-up used in this study, on the other hand, is impossible to seal from the environment completely. This fact per se is not problematic because contaminations on the TL usually occur in one local spot and if e.g. bacteria are in the medium, only a minority can be found in the biofilm. Furthermore, bacterial growth is low because the growth medium optimally does not contain any organics, which would enhance bacterial growth.
The second restriction concerning *Netrium digitus* has to be made here. The large quantities of EPS, which are excreted, present a suitable habitat for many bacterial species. Contamination by bacteria, therefore, is a more problematic risk during EPS production on TL. It cannot be ruled out that bacteria have an influence on the EPS production or may degrade a portion. Therefore this possibility should always be kept in mind. Good reproducibility of growth and EPS production results though indicate that the bacterial influence may not be severe, because it is likely that different bacterial strains contaminated the systems in the independent experiments and thus would have led to different effects because the interaction between bacteria and algae seems to be specific (q.v. Sec.,4.5, p. 87).
Probably the most powerful advantage of the TL system, compared to suspension

culture, is the separation of medium and algae. This results in an increased cell density, which is highly advantageous for harvesting, and the cells are not influenced by the handling of medium exchange in the TL. Two step approaches, but also applications and experiments, where nutrient limitation should be excluded, are considerably easier using a TL system because no separation of medium and cells (e.g. by centrifugation) has to be performed for medium exchange. Viscous cultures are especially laborious to separate from the culture medium and this can only be accomplished by removing the EPS together with the medium. As this was not desirable in the here followed approach, the TL is the only system of the two presented with which the stress experiments were possible to accomplish.

Thinking about future applications and growth of *Netrium* or other EPS producing microalgae reveals more advantages for immobilized cultivation.

First, mixing of liquid cultures already challenges microalgal production (Berner et al., 2015b). The well-known problems will be even more severe with viscous cultures. Raceway or tubular reactors, therefore, are not feasible. The latter would also be expensive in construction and maintenance (Chisti, 2007). Another disadvantage of raceways is contamination risk, as the slow growing algae will be overgrown by faster growing contaminants bound to fall onto the large water surface.

In addition, even if the culture in one or both of the suspension systems had been successful, the cultures would contain mostly water and more energy would have to be invested to separate product from water. The dry matter content in the here presented suspension culture was 1.85 $g \cdot L^{-1}$ and therefore more than one order of magnitude lower than the yield from the TL (2 %). For comparison, the water content of biomass without considerable amounts of EPS grown on TL is only 72-84 % (Naumann et al., 2013).

By using an immobilized system as the TL, the mentioned complications with suspensions can be overcome. First, no mixing will be necessary, only pumping of culture medium. Second, locally occurring contaminations on the growth surface can be removed and thereby are prevented from spreading. Contaminations in the medium may be shielded from the culture by using microporous substrate material. The greatest advantage will be the separation of medium and product, resulting in a cost and energy saving harvest. From a technical point of view, it can be concluded that the TL, which still has room for improvement concerning this particular approach, is by far the best system for cultivation of EPS producing species.

4.2.2 Growth and EPS Production of *Netrium digitus* in Two Different Systems

Analysis of *Netrium digitus* in suspension culture was already undertaken at the beginning of this chapter (p. 63). Here, the comparison to immobilized cultivation should be discussed on the basis of the suspension culture data and the TL cultivation without medium change. Apart from the previously discussed differences caused by the systems set-ups, very specific differences in performance of *N. digitus* in these systems have to be pointed out.

The cell density in the TL system developed differently than in suspension culture. At the beginning of TL cultivation, a certain minimal cell density has to be applied to circumvent a long lag phase or even death of all cells. In all *N. digitus* TL systems this inoculation density was between 3.5 and 5.5 $g \cdot m^{-2}$. For one lab scale TL with 30 filters with a growth area of 18 mm in diameter each, this amounts to approximately $5.2 \cdot 10^5$ cells on this TL, which is supplied with 1 L of growth medium. In a suspension culture with $3.8 \cdot 10^5$ cells per liter, only 73 % of the starting cell number as on a TL was used. Therefore, in the beginning, each cell in the suspension culture had more nutrients at its availability than in the TL. This circumstance changes either when the medium is exchanged regularly or when regular samples are taken from the TL.

In TL cultivation, three filters were removed per harvest, and it was possible to run the system until all filters were harvested. The volume of medium was not reduced while the biomass was removed. In contrast to this, samples of

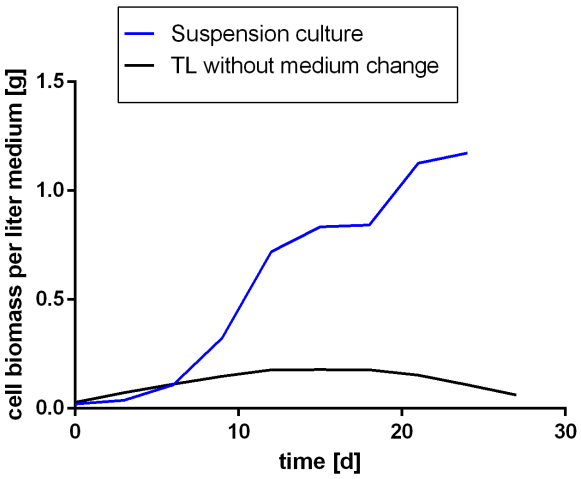


Figure 4.1: Calculated biomass per liter of growth medium in suspension culture and TL over time based on the measured cellular biomass

the suspension culture always consisted of biomass and medium. The ratio of biomass to medium is only altered by growth and not sampling. Therefore, after about 6 days of culture and 2 samples, more nutrients are available per cell (or unit of biomass) in the TL system than in the suspension culture. This also implies that the TL without any medium exchange was not nutrient limited during the culture period of 27 days, which is supported by the strictly linear cellular biomass increase. A graphical representation of this dynamic can be found in Fig. 4.1. Due to the lack of limitation in the TL cultivation, the EPS/cell dry weight ratio diverged between suspension and TL culture after day 9. While the ratio varied around 0.178 for the TL it increased exponentially in the suspension culture to 0.68. A reason apart from the nutrient limitation certainly was the regular homogenization of the suspension cultures before harvest. The remaining cells in the flasks regenerated their destroyed EPS networks after each harvest and thus increased the EPS further. Increased EPS production due to shaking of the suspension cultures was also proven for *Penium margaritaceum* (Domozych et al. (1993) and Tab. 4.2). The biofilms on the TL were never disturbed and therefore had no need to regenerate their EPS networks.
Results of EPS concentrations on TL under a comparable nutrient situation as late in the suspension culture are still pending.

4.3 Growth and EPS production of *Netrium digitus* on Twin-Layer

While growth and EPS production of *Netrium digitus* were only monitored under one set of conditions in suspension culture, the TL system was used to evaluate further conditions and their influence on the two named factors. The focus was laid on the growth medium composition, but also the factors light and substrate layer material were investigated.

4.3.1 Influence of Substrate Layer Material

The change from polycarbonate filter to self-made filters from sawascreen® filter material was not only for technological reasons but also a question of cost.
Polycarbonate filters have proven to be very suitable for lab-scale TL systems for various microalgae. The average pore size of only 0.4 µm is big enough to ensure

good water and nutrient transport, but shields the biofilm from contaminations, e.g. bacteria, from the medium side. The thickness of the filters is low and they easily adhere to the source layer. In addition, the constant weight of the filters makes it convenient to work with. All these advantages and good quality come with a cost. Approximately 0.67 € per filter (catalog price VWR International GmbH, www.vwr.de) is a considerable amount, not only for lab scale experiments but especially when future applications of larger scale are considered. In this case, sheets of at least $1 \cdot 1.5$ m would be necessary, which would be too expensive for most products and are, at this point, not even produced.

The company Sandler (Sandler AG, www.sandler.de) produced a special variant of their sawascreen® filter material (polypropylene-based) fulfilling many of the wanted expectations of a substrate layer. The non-woven fleece is only a few hundred micrometers thick, hydrophilic and adheres to the source layer. Unfortunately, the pore size of 20 µm is not only too big to protect against contamination but also too big to prevent smaller microalgae from entering the medium. Fortunately, *Netrium digitus* is around 220 µm long and 44 µm in diameter and therefore cannot pass the material. An additional advantage is the non-woven structure of the material. The surface is considerably less smooth than the polycarbonate filters and gives the microalgae an additional support structure perpendicular to the substrate material, which resulted in more stable biofilms.

Growth of *Netrium digitus* was one tenth less on sawascreen® material than on polycarbonate (q.v. Fig. 3.5a) which may be caused by the less intense adhesion of the material to the glass fiber. It was observed that individual sawascreen® filters needed to be pressed on the source layer with forceps before they adhered at all points. This situation never occurred with polycarbonate filters and may have resulted in slower water and nutrient support with the filter material.

In the later stage of the cultivation, EPS concentration increased at a significantly higher rate on SC material than on PC (q.v. Fig. 3.5b, p. 36).

The SC material has three clear advantages for *Netrium* cultivation. First, with focus on future large-scale applications, the price per square meter of material is marginal compared to fine-porous polycarbonate materials. Second, the mentioned stability of the culture in the vertical orientation is greatly enhanced. This leads to more reproducible results and makes longer cultivation periods possible. And last, the EPS concentration was close to twice as high on this material.

4.3.2 Influence of Light Source and Intensity

The influence of different wavelengths of light on growth of microalgae is well known (e.g. Glemser et al. (2015); Prokop et al. (1984)). Since different light sources have individual spectra, the light source was also taken into consideration as a possible factor affecting the mucilage amount.
This assumption was based on several findings in literature. Boney (1980) first published an absorption spectrum of *Mesotaenium*'s gelatinous strata showing that absorption is maximal at 400 nm (400 - 700 nm were measured). This discovery was supported by Lütz et al. (1997), who have measured strong absorption in the UV-B range by mucilage produced by *Micrasterias denticulata* and speculated that the mucilage protects the alga from excess radiation of the absorbed wavelength. A similar conclusion was made by Pichrtová et al. (2013), who have investigated *Zygnema*. A strain with a particular thick mucilage sheath also showed low intracellular pigment content, which was assumed to be the primary protection against irradiation.
That a certain mode of mucilage production is influenced by light of specific wavelengths was investigated by Nossag and Kasprik (1993). The orientation of desmid cells in the direction of the light source is common and accomplished by mucilage secretion. Blue (400-480 nm) and to a smaller extent red (660-700 nm) light trigger this movement. The minimal light intensity necessary for the reaction depends on the investigated strains.
The fraction of the EPS, secreted for orientation to the light source, of the total EPS amount is unclear and may only be minor. Nevertheless, these findings lead to assume that the light source might have an influence on the mucilage amount. Also, no studies have been published addressing this circumstance. Because the suspension culture was irradiated with fluorescent lamps and the high light intensities for the TL experiments had to be generated by sodium discharge lamps, possible differences caused by these light sources, with their different spectra, were investigated.
Growth was significantly higher under sodium discharge lamps than fluorescent lamps, while EPS production was not affected by the light source. The, by only 1-2 ℃ increased, temperature in the set-up with sodium discharge lamps probably has caused this increased growth rate.
Apart from the light source, its intensity is of much higher importance and its influence is proven. Microscopical observations revealed enhanced EPS secretion for *Netrium*, when the light intensity under the light microscope was increased (Eder and Lütz-Meindl, 2010). Quantitative results are presented by

Coesel and Wardenaar (1994) for *Cosmarium abbreviatum*. They found that the mucilage sheath's thickness of *Cosmarium* cells strongly increases around cells grown between 0 and 20 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and then is stable up to 120 µmol photons $\cdot m^{-2} \cdot s^{-1}$. Higher light intensities were not investigated. It is possible that EPS production has an equivalent light optimum as known for cell growth. It probably would also be species specific (Coesel and Wardenaar, 1990, 1994; Stamenković and Hanelt, 2013).

By having tested only two light intensities, it cannot be concluded that one of the applied light intensities was optimal for growth or EPS concentration or even both. The higher light intensity (330 µmol photons $\cdot m^{-2} \cdot s^{-1}$) showed significantly higher growth rates and higher EPS concentrations than 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ under otherwise comparable conditions (q.v. Fig. 3.6, p. 37), but if an intermediate or higher intensity had resulted in even better results, stays an open question. E.g. while treatment with 350 µmol photons $\cdot m^{-2} \cdot s^{-1}$ for 1 h already resulted in a strong inhibition of photosynthesis in one *Cosmarium* strain, others were only strongly inhibited at 1 200 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and higher light intensities (Stamenković and Hanelt, 2013).

As mentioned before, virtually no data concerning growth of desmids in immobilized systems is available. The only exception is preliminary data published in a conference paper. Berner et al. (2015a) cultured a *Mesotaenium* strain on a TL. The estimated growth rate was 1.44 $g \cdot m^{-2} \cdot d^{-1}$. Due to lack of replicates and information on growth conditions this value is not reliable.

The here obtained growth rates can be compared with other microalgae, e.g. *Haematococcus pluvialis* (M2826). This alga produces under conditions, comparable to the here tested ones (300 µmol photons $\cdot m^{-2} \cdot s^{-1}$, 1.5 % CO_2), about 10 $g \cdot m^{-2} \cdot d^{-1}$ (Kiperstok et al., 2016). When cell and EPS biomass of the *Netrium* culture with mSFM pH=6 at 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$ are combined a total biomass growth rate of 5.48 $g \cdot m^{-2} \cdot d^{-1}$ is achieved. The 45 % slower growth can be attributed to the finding that desmids from oligo- or mesotrophic habitats show lower growth rates than in cell size comparable species from eutrophic habitats (Coesel and Wardenaar, 1990). And in general, large cells, to which *Netrium digitus* certainly has to be counted, grow slower than smaller cells (Banse, 1976).

4.3.3 Medium Exchange

The TL enables easy medium exchange, which is a major advantage if cultivation without limitation is desired. With other microalgae, medium exchange resulted in higher growth rates than under limiting conditions. The case is different

for *Netrium digitus*. While growth was enhanced, EPS concentration increased less than when the medium was exchanged (Fig. 3.5, p. 36). According to the calculation on p. 70, no nutrient limitation occurred under these conditions. Microalgae adapt to their environment by secretion of substances (Fogg, 1966). In the culture without medium exchange, this conditioning was only necessary once, while in the culture where the medium was exchanged, this process was repeated after every medium exchange and resulted in lower growth rates and higher EPS production. The EPS production, thus, can be seen as a part of the adaptation, as it was also shown by (Surek, 1982). The lower growth may have arisen from lag phases after each medium exchange, but since sampling coincided with medium exchange, these lag phases were not detected.

4.3.4 Influence of Growth Medium Composition

Waris-H (3V) is the standard medium in the CCAC for culturing Zygnematophyceae, but SFM is also frequently used for cultivation, especially in the isolation process of field samples. Both growth media were tested in suspension culture and the results were already discussed earlier (p. 63).
For TL application, the classical SFM was modified by replacing the HEPES with a phosphate buffer, composed of two phosphate salt components, and increasing the total phosphate concentration to the level in Waris-H. This change decreased the N/P ratio from 58.3 to only 5.1, whereas in Waris-H it is 14.3. Because carbon dioxide was supplied to the gas phase, the disodium carbonate, that is included in SFM, was omitted. Resulting from the very different composition, the specific conductivity of mSFM is 40 % lower than in Waris-H with 178 and 307 µS·cm^{-1}, respectively.
Limitation by the nutrients nitrogen and phosphorus can be excluded during TL cultivation. Measurement of the remaining phosphate in the culture medium in the experiment with mSFM at pH=7 and 70 µmol photons·m^{-2}·s^{-1} showed that maximally 36 % of the available phosphorus had been used at the time of medium exchange. Higher growth, as observed under high light conditions, also did not cause limitation because the medium was exchanged more frequently.
Comparing the performance of *N. digitus* on TL with the two media showed no significant differences in growth rate. It has to be mentioned though that the time period of linear growth was shorter with mSFM, both with pH 6 and 7, than with Waris-H 3V. The cellular biomass concentration on day 18 was therefore with 33.05 g·m^{-1} for mSFM and 41.79 g·m^{-1} for Waris-H 3V

significantly different. Both mSFM cultures reached a biofilm thickness of around 5 mm at the time when growth ceased. Limited gas exchange may have caused the observed decrease in growth. Entrapped gas bubbles in the biofilms were regularly observed (cf. Fig. 3.3c, p. 34) and Lehman (1978) and Chang (1980) were able to prove the decrease in carbon dioxide uptake by cells covered by a mucilaginous sheath. Since the majority of the cells were at a low depth of the biofilm, close to the gas phase, this circumstance cannot cause complete cessation of growth. A second possibility could be limited nutrient transport through the biofilm. To gain more insight into the gradients within these thick biofilms, microsensor measurements as performed in Li et al. (2015) (Li et al., 2016) would be very helpful.
The EPS concentrations over time developed very similar with Waris-H and mSFM pH=7. A much steeper EPS increase was observed with mSFM pH=6. This shows the influence of the pH, which is discussed in the following section.

pH of Growth Medium

After the first run with mSFM (pH=7, 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$), this medium was further modified in order to evaluate the influence of pH on the performance of *Netrium digitus* on TL. By changing the proportions of hydrogen phosphate and its conjugate acid, pH values can be set between 5.8-8.0 without changing the total phosphorus concentration in the medium. The conductivity of the media also only differed by 3.4 % and therefore another possibly influential factor was kept constant. Other possible buffer systems, as e.g. sodium acetate/acetic acid, contain organic compounds and thus were not considered due to the risk of increasing bacterial growth in the open system.
Growth rates were higher at pH=7 and 4.5, but the linear range was limited to day 9. As mentioned previously, the thickness of the biofilm limits the obtainable cell dry weight. Hosiaisluoma (1976) stated that a pH of 4.5-5.5 is optimal for the there investigated *Netrium* strain, which fits well with the growth data at this pH. The equally high growth at pH=7 does not fit into the picture but may be explained by the inoculation culture, which was grown in Waris-H 3V at a pH of 7. Therefore, no adaptation to another pH was necessary.
Focusing on the EPS concentration and the EPS/cell dry weight ratio a clear correlation with the pH can be made out. The lower the pH, the more EPS is produced. For two pH values (6 and 7) this was also observed at low light, where the effect was even more pronounced (Fig. 3.6b, p. 37). "Slimy envelopes surrounding the cells were also vaguely discerned" by Hosiaisluoma (1976) during

his study of *Netrium* growth at low pH. In all cases, the correlation of pH and EPS is strongest when the EPS per cell is considered. The highest of all TL EPS/cell ratios was measured at pH=4.5 (0.61).
While the EPS concentration at pH=4.5 resulted in the thickness of the biofilm, the hydration of the EPS at pH=7 may also have played a role in increasing the thickness.

Nitrogen Concentration

After the influence of material, light and pH has been discussed, the question if and which nutrients affect EPS production is addressed here. At this point, the big advantage of the TL system should be stressed once more. The separation of biomass and medium allows easy and frequent medium exchange and change. Thus, limitations of one single nutrient species can be applied while keeping all other nutrients close to the original concentration. This was done for nitrogen, where not only limiting but also abundant conditions were tested.
Growth did not change significantly with changing nitrogen supply. Only after more than 8 days without nitrogen, the cellular biomass did not increase further. Intracellular nitrogen reserves were sufficient to support metabolism until said time. Microscopical examination of the biofilm on day 12 (Fig. 3.10, p. 41) indicated the stress condition before the cellular biomass measurements did. On the other hand, the biofilm and the cells, supplied with additional nitrogen, were of bright green color and several millimeters thick.
Because nutrient limitation in the suspension culture coincided with EPS production and this also is the common opinion in the scientific literature, it was surprising to observe a positive correlation between nitrogen concentration and EPS concentration. After six days of treatment (12 days of culture) 25.02 and only 5.44 $g \cdot m^2$ EPS were measured for high and no nitrate, respectively.
A similar effect by nitrate was observed by Domozych (2007) with *Penium margaritaceum* cultured in variations of Woods Hole Medium. *P. margaritaceum* cells also produced more EPS when more nitrogen, in form of nitrate or ammonium, was added to the medium. Domozych (2007) hypothesized that the cells produce the EPS in order to sequester the additionally provided nitrogen.
The control treatment with additional salt was intended to exclude the possibility, that the increase in conductivity, which inevitably accompanies the increase in nitrate, influences the EPS production of *Netrium* rather than the nitrogen. Growth and EPS production showed interesting results for this treatment. While growth was enhanced to 4.87 $g \cdot m^{-2} \cdot d^{-1}$, the EPS concentration, as well as the EPS/cell dry weight ratio, was lower than in the high nitrogen treatment and

equal to the standard conditions. The EPS/cell dry weight ratio showed that the ratio is lower with the addition of salt than under standard conditions. This can be attributed to the high growth rate. Interestingly, the slope of the ratio is strictly linear in contrast to all other EPS/cell ratios. The conclusion can be made that nitrogen and not conductivity caused the increase in EPS.

Conductivity

The influence of conductivity was already considered within the nitrogen treatments. Apart from salt addition, the conductivity can be also altered by diluting the nutrient concentrations equally. By exchanging the medium every two days no limitation for the majority of the nutrients should have occurred.
Enhanced growth in biofilms supplied with 1/2 mSFM compared to full strength mSFM corresponds well with observations made during isolation of desmids from field samples (M. Melkonian, personal communication). Switching to 1/4 mSFM after six days, a point where some EPS was already produced, had a drastic effect on the biofilm. Growth, as well as EPS production, ceased and in addition the present EPS swelled, thereby developed a more liquid consistency and eventually caused sliding of the biofilm. The loss in stability of the EPS was caused by the insufficient amount of ions in the 1/4 mSFM. These associate with or cross-link the anionic residues and stabilize the structure.
The negative growth rate and the stable EPS concentration allows the conclusion that no cellular biomass was produced anymore, but EPS may still have increased. This increase was not measurable, because the biofilm spread over a larger area and was, when the original growth area is considered, diluted. EPS production and spreading over a larger area counteracted and resulted in a stable EPS concentration per area.
The cross-linking or association argument is also congruent for the salt treatment in which the biofilm stability was exceptionally high and a maximal thickness of 10 mm was reached. Together with the control experiments, in which the biofilm had an intermediate stability, a connection between conductivity and biofilm stability can be recognized. Increased viscosities of polysaccharides at higher ionic strengths is a commonly observed phenomenon not only with divalent ions but also sodium chloride (Smidsrød and Haug, 1971; Wu et al., 2011; Tuvikene et al., 2015). The EPS per cell ratio is a factor which should also be taken into consideration. In many cases, a high stability was observed where this ratio was low.

4.3.5 The Biological Role of EPS for Desmids

The preceding discussion focused on some biotechnological aspects of EPS production. Nevertheless, it also contained some hints concerning its biological function for *Netrium* and zygnematophyceaen algae in general. Here, these aspects are summarized.

The secretion of resources bears a higher risk of loss than intracellular energy investments, but this risk must be outweighed by the benefits, otherwise this phenomenon of secretion of microgram quantities of EPS per cell would not have evolved. Thus, the high amounts of polysaccharides, which are secreted by desmids, give reason to believe that its function or likely functions are of major importance to these algae.

One factor clearly affecting EPS concentration is the light intensity. The data presented here give quantitative results on the EPS concentration under different light regimes. In accordance with literature findings (Eder and Lütz-Meindl, 2010; Coesel and Wardenaar, 1994) it can conclusively be proven that more mucilage is produced per cell dry weight at higher light intensities. The role of the mucilage likely is not a protection mechanism against excess radiation or particular wavelengths, because the used light sources did not emit any considerable radiation below 400 nm, below which absorbance by the mucilage occurs and the protective role was assigned to (Brook, 1981). The protective character may lie in the prevention of desiccation, which is more likely to occur at higher light intensity, at which the temperature is also likely to increase. Especially for terrestrially occurring species, an effective protection against dryness is of great importance. Studies have shown that the aeroterrestrial-living *Zygnema*, a phylogenetic ancestor of desmids, produces thick mucilaginous sheaths, which reduce water loss (Pichrtová et al., 2014).

Enhanced EPS secretion at higher nitrogen availability was observed in this study and also by Domozych (2007). The function of EPS likely is not connected to the uptake of the provided nitrogen, as hypothesized by Domozych (2007) because then higher growth should have been observed, which was not the case in neither of the studies. It is more likely that other, positively charged nutrients, are sequestered and then taken up. It can be assumed that excess nitrogen gives the algae the possibility to produce more EPS for this sequestering function because more protein and therefore enzymes can be produced. At lower nitrogen concentrations the EPS production is to some degree nitrogen limited, even though the EPS itself does not contain detectable amounts of protein nor aminated monosaccharides, but nitrogen is needed in the mucilage production

process.
Due to the high uronic acid content of the EPS, it has anionic properties and studies have directly shown that it has a strong binding affinity for positively charged molecules e.g. calcium ions (Eder and Lütz-Meindl, 2010). The destabilization of the biofilm grown at 1/4 mSFM also supports the function of binding ions.
In the nutrient scarce habitat of peatlands, in which desmids are commonly found, mucilage, which also provides an increased surface area of the cell, can bind ions and trap them in the immediate vicinity of the cells. Specific uptake mechanisms by counter ion transport or antiport then enable nutrient uptake. Capture of iron, a frequently limiting micronutrient for plants and microalgae (Coale et al., 1996), and potassium, which is highly mobile in peatland waters (Rydin and Jeglum, 2013), are just two examples how EPS can benefit the microalgae.
Another possible function of EPS, which can be seen as an addition to the previously elaborated function, is the creation of a biovolume enhancing environment around desmid cells in which alkaline phosphatases are bound and can hydrolyze organic phosphorus (Spijkerman and Coesel, 1998). Both functions, ion capture and phosphatase activity, are adaptations to the oligotrophic habitats of desmids.
No quantitative data, apart from this study, nor explanation of the effect of pH on the EPS concentration of any Zygnematophyceae are available. It can be speculated that the cells need more EPS because less of the anionic groups of the present polymers are dissociated at low pH and therefore they are less effective at e.g. binding cationic moieties.
Algae are never occurring isolated from other organisms. The interaction with other microorganisms, especially bacteria, and the role of EPS in it shall not be forgotten at this point. A detailed discussion can be found on pp. 87.

4.4 EPS Composition

4.4.1 Methodology

The three steps: sample preparation, hydrolysis and chromatography were implemented and optimized fitting the requirements of *Netrium* EPS samples.
The analysis of the hydrolysates by high-performance liquid chromatography (HPLC) with prior derivatization was achieved using 50 mM sodium phosphate

buffer (pH=6.5) with an ACN concentration between 15 and 21 % and an UV-Vis detector. While the derivatization procedure from Dai et al. (2010) was only modified in one step, the mobile phase gradient was successfully optimized for the here used Sherisorp ODS 2 C18 reversed phase column. Adaptation was necessary because stationary phase specifications as pore size, surface area or carbon load vary between C18 reversed phase column products and result in differences in separation efficiency.

The chromatogram of the monosaccharide standards showed that compositional analysis is possible with reversed phase C18 Sherisorb ODS2 and the Merck-Hitachi L-6200 system. By using an internal standard, variations were minimized. The internal standard cannot only be used to eliminate differences in injection volume but also to estimate the shifts in retention time which may occur by using different batches of mobile phase and by temperature changes during analysis (Stepan and Staudacher, 2011). By analysis of standard samples, changes in separation performance were excluded and possible differences in derivatization efficiency were compensated.

The aforementioned modification of one derivatization step was introduced at PMP extraction. It was observed that addition of buffer (50 mM sodium phosphate buffer pH=6.7) increased the stability of the PMP-monosaccharides in comparison to the addition of water. Samples in water showed lower peak areas after 24 h, while buffer addition resulted in an unaltered response. This optimization was only a safety measure because the samples were always measured within ten hours after derivatization, but may be of interest if the analysis has to be postponed.

After chromatographic separation of the expected monosaccharides was implemented, hydrolysis conditions were investigated.

A complete recovery after hydrolysis of the applied dry weight was never achieved. Total yield ranged between 38 and 75 %. These values are in good agreement with Prieto et al. (2011), who reached a maximal recovery of 58 % among 120 different tested conditions. The effects underlying these losses in recovery are degradation of hydrolyzed monosaccharides and possible reversed hydrolysis. De Ruiter et al. (1992) have shown that uronic acids, as well as mannose monosaccharides, are more susceptible to degradation during hydrolysis with TFA than other free monosaccharides. In *N. digitus* EPS, glucuronic acid was present in large quantities, but degradation of mannose might have been crucial for the detection of this sugar, which was only present in low concentrations or, as in the case of the hydrolysis optimization samples, not detected at all.

Further, it was observed that 4 mg·mL^{-1} EPS for hydrolysis, as recommended by Dai et al. (2010), only resulted in approx. 25 % of recovery whereas 10 mg·mL^{-1} reached the stated average of 55 %. The higher EPS concentration for hydrolysis also had a positive effect on the detection of monosaccharides of lower quantity as mannose and galacturonic acid, which were only detected when 10 mg·mL^{-1} had been used.

While the higher TFA concentration of 4 M was clearly superior, two arguments led to the selection of 4 hours at 110 ℃ as selected hydrolysis conditions over 8 h at 80 ℃. First, the glucuronic acid content was higher in the former. Since uronic acids are a characteristic and property giving group of the investigated EPS, their content should not be underestimated while the loss of e.g. xylose at the chosen conditions was judged to be less critical because the quantity was still high. Second, the standard deviation for the percentage of applied EPS was much lower at 4 h and 110 ℃ than at lower temperature but longer time (cf. Fig. 3.15, p. 47).

The development of recovered percentages of each monosaccharide with increasing hydrolysis temperature and duration (Fig. 3.15, p. 47) showed which components were most susceptible. It further can be concluded that the non-recovered fraction most likely contains these easily degradable monosaccharides. Losses in xylose and the unknown component were most prominent. Arabinose and galactose also showed losses with increasing temperature and time.

An EPS isolation protocol consisting of centrifugations, precipitation and dialysis was used by several researchers (Kiemle et al., 2007; Domozych et al., 1993, 2005). Domozych et al. (2005) even suggested that "the relatively simple methods designed for extraction of EPS to date, may provide the basis for a consistent protocol for EPS isolation, characterization and comparative biochemistry". Comparison of dialyzed and precipitated EPS from *Netrium* showed no differences in monosaccharide composition. A combination of these two purifications was used for the compositional analyses. After isolation, the EPS were precipitated and dry weight was determined since these precipitates have a purity of 98 % polysaccharides (Domozych et al., 2005). The following dialysis did not lead to considerable reduction of sample dry weight, but to an increase in recovered percentage after hydrolysis, which indicates higher purity.

The glucose content in the non-treated EPS was reduced by precipitation and dialysis; it, therefore, was not solely of polysaccharide origin.

During the implementation of the analysis conditions, the focus was directed to yield together with reproducibility of results. The selection of the hydrolysis conditions was the most critical point as they greatly influenced

the monosaccharide composition. Purification and chromatography only had little to no influence. After the evaluation of the methodology was completed and during the analysis of samples, derived from experimental cultures, a further improvement was achieved. Compared with results of the hydrolysis optimization (Fig. 3.14b, p. 46) the total recovery from TL and suspension culture samples was at least twice to more than three times as high. The reason lies in the milder drying of the hydrolysates. The milder drying, which together with the higher EPS concentration for hydrolysis (discussed earlier), resulted in the detection of more monosaccharides. Mannose and galacturonic acid were only detected when these conditions were applied. This should not be interpreted as a change in composition between samples used for hydrolysis optimization and from experimental cultures but can be fully traced back to the improved methodology.
All results from suspension and TL cultures discussed in the following section were obtained applying identical methods and are therefore fully comparable.

4.4.2 Comparison of Composition

During suspension culture of *N. digitus* for growth and EPS production kinetics, samples for monosaccharide compositional analysis were also taken. The sample from the late exponential phase and from the stationary phase only differed in the arabinose content, which was higher in the former sample (cf. Fig 3.17, p. 49). Compositional analysis of EPS produced by *Netrium digitus* was conducted before. Three research groups using three different strains and three different methods have published their results until now. In all cases, material from suspension cultures was used. Thus, comparison with averaged results from the suspension culture is most reasonable. As the hydrolysis optimization showed, the compositional output varied considerably when conditions were changed. Since the results found in scientific literature, and which are summarized in Tab. 4.3, were obtained under different conditions, they have to be compared with some caution.

In general, the compositions have similar characteristics. Either xylose, as in the case for this study and the EPS isolated by Kattner et al. (1977), or fucose (Kiemle et al., 2007; Eder and Lütz-Meindl, 2010) are the major components. Arabinose, rhamnose, galactose, glucose and mannose are present in lower quantities. The here measured uronic acid content is comparable with Kiemle's value, but higher than in Eder and Lütz-Meindl's sample.

Table 4.3: Monosaccharide composition of *Netrium digitus* EPS in this study and in literature, expressed in spot intensity (+ to +++) or as percentage of total analyzed carbohydrate; values by Kiemle et al. (2007) were recalculated to incorporate the spectrophotometrically measured uronic acid content(*); GC-gas chromatography, TLC-thin-layer chromatography

	Kattner et al. (1977)	Eder and Lütz-Meindl (2010)	Kiemle et al. (2007)	this study
method	TLC	GC	GC	HPLC
Arabinose	+	7	7.7	5.87
Rhamnose	+	5	7.7	2.44
Xylose	+++	20	13.7	39.36
Galactose	+	12	7.8	3.07
Glucose	+	13	6.4	3.44
Glucuronic acid	+	4	15.6*	14.24
Galacturonic acid	+	3		0.29
Fucose		30	38	17.25
Mannose		6	3.0	0.4
Ribose			0.2	0.0
unknown	3 different			13.65
Sulfate			3	

A methodological influence may be seen in the high glucose concentration in the sample of Eder and Lütz-Meindl (2010). This sample was not dialyzed and comparison of EPS treatments (p. 48) has shown that the glucose concentration was significantly influenced by purification. On the other hand, especially the large differences in xylose and fucose content within samples make a methodological bias unlikely to be the cause of the differences in composition between the *Netrium* strains. Therefore, the composition most likely is strain specific within the range of common features.

In this study, one component, eluting after 16 min, was not identified by standards. Interestingly, no possible match was found in the literature on this topic. Ribose eluted closer to glucuronic acid than the observed unknown peak and was therefore excluded as the cause. Further deductions concerning the identification of the unknown component can be made. First, the orientation of the hydroxyl groups in positions 2 and 3 of the sugar molecule can be determined, because the elution order of PMP-monosaccharides is primarily determined by this orientation. A *cis*-orientation correlates with early elution

and a *trans*-orientation with late elution (Strydom, 1994). Furthermore, charged molecules, as uronic acids, also elute fast. The unknown therefore might be another uronic acid (e.g. mannuronic acid or guluronic acid) or a hexose or pentose with carbon atoms 2 and 3 in cis-orientation, viz. lyxose, allose, gulose or talose. The elution of guluronic acid, as well as gulose, between mannose and ribose was confirmed in comparable systems (Strydom, 1994; Stepan and Staudacher, 2011).

The comparison of the compositions presented in Tab. 4.3 and a closer look into the connected literature give arguments for the possibility of the unknown component being of acidic nature. As Kiemle et al. (2007) measured uronic acids by a colorimetric method and not by chromatography, the value of 15.6 % would include e.g. mannuronic acid or guluronic acid without identifying them.

A similar possibility was considered by Eder and Lütz-Meindl (2010). They determined glucuronic and galacturonic acid by gas chromatography and pointed out the surprisingly low uronic acid content. They hypothesized that further uronic acids might have been present in the mucilage, which were not detected due to lack of standards.

A further possibility of the identity of the unknown component can be the substitution of reactive groups to monosaccharides already detected, which would also alter the retention time. A possible substitution may be sulfatation, which was measured by Kiemle et al. (2007) to be 3 % in *Netrium digitus* EPS samples. The binding of antibodies, raised against methyl-esterified pectins, to mucilage of *Micrasterias denticulata* suggests esterification of the carboxyl group of the uronic acids (Eder and Lütz-Meindl, 2008). Further antibodies also labeled N-acetyl-galactosamin, N-acetyl glucose and -galactose (Kiemle et al., 2007). Domozych et al. (2005) detected around 5 % methylated sugars in polysaccharides of *Penium margaritaceum* and therefore methylation should also be considered as a likely modification. Even though the organism is phylogenetically more distant, EPS of *Rhodella grisea* contain acetyl and succinyl groups (Capek et al., 2008), which would be further possible substitutions.

Sulfated and methylated sugars withstand the hydrolysis procedure and can be detected with the applied method (Strydom, 1994; Neeser and Schweizer, 1984). The attempt to identify the unknown compound by mass spectrometry was not successful until now.

More data on the monosaccharide composition of EPS, produced by other desmids, is available in literature (Domozych et al., 1993; Lombardi et al., 1998; Domozych et al., 2005; Giroldo et al., 2005; Kiemle et al., 2007) as well as in Chapter 3.4.3 (p. 56), in which the composition of two *Cosmarium* species

was analyzed. The comparison of the different sources reveals that differences between species of the same genera are as large as between phylogenetically more distant groups. Thus, no pattern fitting the phylogenetic tree can be made out on the monosaccharide level. Nevertheless, the data permits the conclusion that fucose, xylose, galactose and uronic acids are the most abundant monosaccharides. Usually, two of the aforementioned monosaccharides constitute over 50 % of the total carbohydrate fraction. The remaining amount is divided between six to seven other monosaccharides whereas mannose and ribose are only found in minor quantities.

The high number of different monosaccharides in the EPS of desmids is comparable to the situation in diatoms as the summary presented by Hoagland et al. (1993) shows. Dominant sugars are fucose and rhamnose and higher sulfate concentrations were measured compared to desmid EPS. Fewer components are present in Ulvan produced by *Ulva* species, which contains six different monomers, including the rare uronic acid iduronic acid, and up to 30 % of the monomers are sulfated (Quemener et al., 1997). Even less diversity is found in the EPS of *Porphyridium* sp., which is mostly composed of xylose and every sixth monomer is sulfated (Geresh et al., 2009). The situation gets even more monotonous when looking at the polysaccharides produced by red and brown macroalgae. The well-known polysaccharides, such as agar and carrageenan, mostly contain galactose (Melo et al., 2002; Jiao et al., 2011). Fucoidan, a polysaccharide produced by brown algae, only consists of fucose and terminal glucuronic acid residues (Bilan et al., 2002).

Little is known about the variability in monosaccharide composition caused by culture age or modification of the culture conditions. The results presented in Fig. 3.17 (p. 49) show that variation is minimal between EPS secreted in the exponential growth phase compared to the stationary phase. Only the arabinose content was significantly reduced between day 12 and 18. Comparable results were obtained by Domozych et al. (2005), who have found significantly less arabinose and glucose in six months old soluble EPS compared to six weeks old samples of the EPS from *Penium margaritaceum*. The arabinose content in TL EPS was similar to the one of the late suspension culture EPS. The presence of mannose in all analyzed samples correlated with this low arabinose content. The difference in glucose content, observed by Domozych et al. (2005), was not found in the suspension culture samples, but a clear difference of 51 % less glucose in TL EPS compared to suspension was observed. The xylose content was also elevated in suspension culture EPS, but only by 5 %. In contrast to these monosaccharides, the deoxy sugar fucose was more prominent in TL

EPS (33.3 % more). Adhesion of cyanobacteria is attributed to hydrophobic interactions (Fattom and Shilo, 1984), which can be achieved by deoxy sugars with hydrophobic characteristics. In fact, Wustman et al. (1997, 1998) have shown that the polymeric stalks of the diatom *Achnanthes longipes*, which enable adhesion to solid substrates, consist of more than 20 % fucose. Fucose was also elevated in diatom EPS produced under phosphate and nitrogen limitation (Abdullahi et al., 2006; Magaletti et al., 2004), which was also attributed to adhesion activity. Under said conditions, glucuronic acid was also increased by up to 113 %. A similar development was not found with *Netrium* EPS, produced in suspension nor immobilized, but the glucuronic acid content was increased by 14.1 % at low light compared to high light.
It can be concluded that immobilized and suspended cultivation, as well as light intensity, influence the monosaccharide composition to some degree. In all cases, the order from highly to less abundant monosaccharides was never altered. The function of the EPS, apart from adhesion, probably exerts its influence more by the amount than by the composition.

4.5 Interaction of Desmids and Bacteria

In order to interpret the relationship between the present bacteria and the investigated algal species correctly, it has to be emphasized that all algal strains have been in the CCAC for years before the growth experiments were conducted. This allows the conclusion that harmful effects by the bacteria can be excluded, since they would have made the effortless cultivation of the algae impossible. It was shown that the bacterial community associated with algae is specific and also different from the surrounding water body (Lachnit et al., 2009). A specific association without any interaction at all is highly unlikely and the lack of any considerable organic carbon source in the algal medium makes the bacteria dependent on the carbon fixed by the algae. Since harmful effects were excluded, based on the grounds of successful yearlong cultivation, the interaction likely is of positive nature.
The growth rates of all six co-cultures of *Cosmarium humile* as well as *C. pachydermum* with *Pseudomonas moraviensis* were slightly, but significantly higher than in the axenic control. In all cases, the bacteria also showed distinct growth during the exponential growth phase of the microalga. The axenic culture also grew and reached comparable final cell numbers but growth was slower in the first 14 days. The comparable final cell numbers indicate that the co-cultured

bacteria did not mobilize any nutrient resources, which are not accessible by the microalga alone because this would have led to lower cell numbers in the axenic culture. The slightly lower final cell numbers in the co-culture of *Cosmarium humile* with the *Cytophaga* strains can be interpreted as a competition for the available nutrients and an earlier limitation for the algal cells.

A different case is presented with the *N. digitus* cultures in Waris-H without soil extract nor vitamins. The growth rate and the cell number after 18 days were lower in the axenic culture and these algal cells also showed signs of stress. Thus, the bacteria in the xenic culture compensated the omitted soil extract. Since Waris-H without vitamins did not cause any limitation for axenic *N. digitus*, vitamin production by the bacteria (Croft et al., 2005) can be excluded as a cause. Growth in SFM, which does not contain any soil extract, was also high for the axenic culture. The alga, therefore, can compensate the missing soil extract or a component on its own. The reason may be found in the mineral composition of the growth medium. A number of micronutrients, which are necessary for cofactors, are supplied with SFM but not Waris-H. In the axenic SFM culture the algae can synthesize all cofactors, because the metals are present, and therefore can compensate the lack of soil extract on their own.

The bacteria co-cultured with the *Cosmarium* strains were identified based on their 16S rRNA gene sequence. The occurrence of bacteria belonging to the Bacteriodetes is common for phycosphere samples (Goecke et al., 2013) and three representatives were found in the xenic *Cosmarium humile* culture. *Cytophaga* is one of these representatives and it was also shown that specific auxin-like growth factor release results in thallus differentiation of *Ulva mutabilis* (Spoerner et al., 2012). *Rhizobium* was also found in multiple samples of immediate microalgal environments and the presence in cultures of *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Scenedesmus* sp., and *Botryococcus braunii* resulted in up to 72 % more algal cells (Kim et al., 2014). The *Rhizobium* identified by Kim et al. (2014) has 96 % sequence similarity (16S rRNA gene) with the *Rhizobium* found in the *Cosmarium* culture. The mechanism of interaction was not investigated in Kim's study, but it is known that *Rhizobia* communicate via quorum-sensing with roots of higher plants (Sanchez-Contreras et al., 2007).

The second representative of the α-proteobacteria found in the culture was *Asticcacaulis excentricus*. One sequence closely related to the here isolated organism was also found in another freshwater ecosystem (Gordon-Bradley et al., 2014). No records of interaction with algae have been found, but the available genome sequence of *A. excentricus* (JGI project ID:4085008) would be a possible starting point for an investigation.

Arcicella aquatica was characterized by Nikitin et al. (2004) who have shown a high activity of β-galactosidase and α-glucosidase.
The isolated bacterial strain in the xenic *C. pachydermum* culture was identical in its 16S rRNA gene sequence with *Pseudomonas moraviensis* strains. The genus *Pseudomonas* is diverse and representatives are found in a variety of habitats. Plant and animal pathogens are common (Beiki et al., 2016; Chastre and Fagon, 2002), but also plant growth promoting effects of e.g. *Pseudomonas putida* were shown (Patten and Glick, 2002). The plant hormone indole 3-acetic acid involved in this interaction is also produced by a close relative of the strain found in the *C. pachydermum* culture (Verma et al., 2015). A positive effect on growth of *Chlorella* sp. and *Scenedesmus bicellularis* was also caused by two *Pseudomonas* species (Mouget et al., 1995). The presence of bacteria in the immediate vicinity of the alga has the advantage that the oxygen produced by the alga is converted to carbon dioxide by bacterial respiration and can then be fixed to organic carbon by the alga. Especially in EPS containing and therefore viscous cultures this enhanced gas exchange may give the observed advantage in growth. This then would be a general effect rather than a species-specific one.
Nutrient recycling is another common type of interaction of algal-associated bacteria (Bloesch et al., 1977). Due to the short time period, in which growth was monitored, this effect was not exploited to its full potential. The observation of several months old xenic cultures in comparison to axenic ones of the same age showed more intense green color in the xenic cultures, which indicates nutrient recycling.
Not only growth and cell division can be enhanced by bacteria. For example, Bruckner et al. (2008) have shown increased soluble as well as bound polysaccharides amounts in co-cultures of *Cymbella microcephala* with different bacterial strains isolated from xenic cultures. Only one of the six investigated bacterial strains, Bacteriodetes strain 32, had a negative impact. Said strain also decreased the amount of carbohydrates in cultures of *Achnanthidium minutissimum* and induced capsule formation of both diatoms (Windler et al., 2015).
The *Cytophaga-Flavobacteria* cluster, to which Bacteriodetes is affiliated, is well known for its ability of high molecular weight dissolved organic carbon degradation (Kirchman, 2002) and a close relative of the *Cytophaga* found in the *Cosmarium humile* culture proved this point (Oppong et al., 2003). The EPS concentrations estimated after isolation from xenic and axenic cultures give reason to believe that degradation by bacteria took place. Since no biological replicates were made, these values are not well supported, but the great differences between axenic and xenic culture of more than 100 % indicate that EPS is affected

by bacteria. Apart from the stated possibility that degradation reduced the concentration, it is also possible, but less likely, that EPS secretion is suppressed in the presence of bacteria. The lack of any added organic carbon (apart from soil extract) to promote heterotrophic growth supports the former scenario.
The evaluation of the monosaccharide composition of EPS isolated from axenic and xenic cultures only showed significant differences in the abundance of the unknown component. For both *Cosmarium* species the content was higher in the EPS produced under xenic conditions. Interpretation of this result is made difficult by the unknown nature of the unidentified sugar. It can be concluded though that either the bacteria degraded the EPS selectively and avoided this component or it was also produced by the bacteria and added to the total of all polysaccharides in the culture supernatant. EPS production by *Rhizobia* is pronounced and the monosaccharides correspond to the composition of algal EPS (Castellane et al., 2015). Similar results were found for a *Cytophaga* strain (Mojica et al., 2007).
EPS from *N. digitus* under xenic and axenic conditions was also investigated. The suspension culture was axenic, while the TL samples contained bacteria. Their abundance and identity was not known and likely varied between cultures and even samples.
An interesting result was the high viscosity of the EPS in xenic cultures even though their concentration was considerably lower. If degradation is once more considered, the resulting change in EPS structure due to the activity of extracellular bacterial enzymes can have caused the higher viscosity. Furthermore, it is also possible that the bacteria themselves or secreted substances, such as proteins or organic acids, functioned as cross-linking agents.

The initial goal of the inhibitory zone tests with algal cultures on pathogenic bacteria was to investigate possible antibiotic effects by the algae and their polysaccharides. This hypothesis was dismissed but the results point into another promising direction. The finding of antibiotic properties of associated bacteria is interesting because this shows another possible positive effect of bacteria in algal cultures. The antibiotic substance produced by the associated bacteria protect not only themselves but also the microalgae.
From the presented results it can be concluded that bacteria have a weak positive effect on growth during the exponential growth phase of the *Cosmarium* strains. The EPS concentration in stationary phase cultures is lower in xenic than in axenic cultures, but the viscosity of the suspensions is higher in the xenic cultures. Complete degradation of EPS in suspension culture was never observed. For the yield in a larger scale production of EPS, degradation by bacteria certainly is an

issue but for reasons discussed on p. 65 closed and therefore axenic systems are not feasible for this purpose. The lab-scale TL, on which EPS production was investigated, is an open and therefore non-axenic system. Complete degradation of EPS was never observed here neither. The extent of partial degradation cannot be measured nor estimated at this point but the results have proven that considerable amount of EPS can be produced under non-axenic conditions. A possible loss of product may be compensated by manipulating the culturing conditions and therefore increasing EPS production. If bacteria prove to have a severe negative influence on EPS yield, conditions of low nutrient content rather than high nitrogen will be of advantage, since nutrient deplete conditions to some extent will also limit bacterial growth. Further, it may also be possible to protect the microalgae from some bacterial species by co-culturing them with bacteria with antibiotic properties as the associated bacteria of *Netrium digitus* and *Cosmarium subtumidum*.

4.6 EPS Quantification with Ruthenium Red

As shown in Sec. 3.5 (pp. 58) RR can be used to quantify EPS from Zygnematophyceae. The originally used conditions of 12.7 $mg \cdot L^{-1}$ and neutral pH give a linear response in dependence of the applied EPS concentration. In order to increase the slope in the assay, nine combinations of RR concentration and pH were tested. Best results concerning minimal standard deviation were obtained at 38.5 $mg \cdot L^{-1}$ and pH=10.68. At these conditions, smaller differences in EPS concentration can be measured more reliably.

The starting point of the development of the assay was the RR adsorption method by Figueroa and Silverstein (1989) in which a RR solution was added to an activated sludge pellet and the absorbance of the supernatant after 3 h of incubation was read spectrophotometrically. The researchers showed that the non-destructive method with RR gave better results than exo-polysaccharide extraction with NaOH or EDTA and quantification with phenol-sulfuric acid (Dubois et al., 1956). Among temperature (4-23 ℃), incubation time (up to 3 h) and pH (5.8-8.3), the latter had the largest effect on the adsorption intensity of RR to sludge particles. The proposed method of adding RR to a pellet and the relatively high reaction volumes of 10 mL per sample were not applicable for the here intended purpose because the extracellular polysaccharides are mostly dissolved in the culture supernatant after centrifugation and lower sample volumes were desired in order to save sample and reagent. Thus, Figueroa and Silverstein's method was modified using liquid samples of small volumes

(first 100 then 20 µL) and a comparably larger RR solution volume (900 and 180 µL, respectively). An incubation period of 3 h, as proposed by Figueroa and Silverstein (1989) was also tested, but no increased response was detected, but the variability of the absorbance measurements was increased. Therefore, immediate further processing (centrifugation) is recommended for the RR method for liquid samples. This difference can be explained by the denser structure of sludge flocs in comparison to dissolved EPS and therefore RR diffusion to some adsorption sites is hindered in the former case.

The here presented assay shows many similarities to a method published by Ortiz et al. (2014). It has to emphasized that the assay development with the original conditions was performed before this publication was available and therefore was independent of its results. The published method is more complex because it involves the enzyme reaction prior to polygalacturonic acid (PGA) quantification. The researchers also recommended small sample volumes and larger RR solution volumes and even the concentration of polygalacturonic acid (0.128 $g \cdot L^{-1}$) and RR (288 $mg \cdot L^{-1}$ for reaction and maximally 36 $mg \cdot L^{-1}$ for measurement) were in the same range as the in this study proposed values. By the addition of 8 mM NaOH Ortiz et al. (2014) probably have had a pH of 11.7 (calculated), which they said reduced background at high PGA concentrations but also is the desirable pH for increased RR adsorption stated by other researchers before.

Comparison of the achieved slopes also shows similarities. The given slope of 0.0088 unit differences in absorbance per applied µg of polygalacturonic acid can be converted to 0.3254 units difference in absorbance per g/L PGA without dilution which corresponds to the unit in which the slopes were expressed in the results section. The converted value lies between the slopes for *C. pachydermum* EPS (0.2367) and *N. digitus* EPS (0.443).

A common observation concerning the adsorption of RR by polysaccharides is the influence of the pH on EPS-RR complex formation. Figueroa and Silverstein (1989), Ortiz et al. (2014) and Santhiya et al. (2001) all observed increased adsorption at alkaline pH. The reason probably lies in the ionized form of the carboxyl groups of the acidic polysaccharides, which are more accessible for the dye (Luft, 1971), even though Figueroa and Silverstein (1989) noted that uronic acids with a pKa of around 3.6 should be fully deprotonated at pH=5.8. Their interpretation was that divalent cations, which bridge the carboxyl groups within the polysaccharide, are taken up by excess hydroxyl ions and therefore free additional adsorption sites.

Higher variability in the absorbance reads at high RR concentrations may arise

from unspecific binding of RR to already formed RR-EPS complexes.

The here presented method for EPS quantification posed to be reliable. Compared to other spectrophotometric methods as the widely used phenol-sulfuric acid assay by Dubois et al. (1956) it has further advantages. The first major advantage is the possible down-scaling to microtiter format. Due to the extremely different volumes of phenol and sulfuric acid, smaller reaction volumes than 3 mL can not be used for the PSA assay. Further, only one chemical is necessary, which in addition does not have many of the toxic and hazardous properties of either phenol or sulfuric acid. The often forgotten bias of this method for certain monosaccharides (e.g. fucose) and complete lack of reaction with uronic acids is also important for the RR adsorption assay. In both cases, zygnematophyceaen EPS are less problematic in this regard because their composition does not change drastically over the culture period or under varying culturing conditions. For both assays, a standard, made of lyophilized EPS of high purity, may improve the results considerably.

5. Summary and Conclusion

This thesis covers first attempts of growing *Netrium digitus*, an EPS producing zygnematophyceaen alga, immobilized on a porous substrate photobioreactor. Important parameters such as medium composition and light intensity were investigated for their influence on EPS production.

The EPS increase over time in an axenic suspension culture of *Netrium digitus* was unmistakably shown. After the exponential growth phase had ended the EPS concentration still increased up to 0.79 $g \cdot L^{-1}$ on day 24. Under the prevailing nutrient limitation, 0.68 g EPS per gram cellular biomass were produced. This is a higher ratio than in all tested TL cultures, where the maximal ratio of 0.61 g EPS per gram cellular biomass was reached with a growth medium with a low pH of 4.5. Although the cells on the TL were never nutrient limited as in the suspension culture, appreciable EPS concentrations were obtained. The highest EPS concentration after only 12 days of cultivation was achieved at 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$ together with the addition of 5-fold nitrogen to mSFM (pH=6) and amounted to 25.02 $g \cdot m^{-2}$. This clearly shows that many possibilities are available to manipulate EPS production and that the strategy has to be adapted to the intended cultivation system.

For the use of the TL technology, the stability of the biofilm has to be taken into consideration. It was shown that addition of salt to the medium increased the stability, but in accordance with the other growth data, cellular biomass, as well as EPS concentration, did not increase once the biofilm thickness reached 5-6 mm.

The problematic of biofilm thickness and therefore stability has to be targeted before upscaling is considered. Also, influences by bacteria have to considered in open systems. Growth data from suspension cultures showed that co-cultured bacteria had a positive effect on *Netrium* as well as *Cosmarium humile* and *Cosmarium pachydermum*, but EPS in xenic cultures were reduced. During TL cultivation no negative effects by contaminating bacteria were recognized.

The analysis of the monosaccharide composition of the EPS showed xylose, fucose, glucuronic acid, an unidentified monosaccharide, galactose, glucose, rhamnose,

mannose and galacturonic acid to be present in decreasing order. This order of abundance was not altered by any of the tested culture conditions, but differences in abundance of single monosaccharides were observed. E.g. TL EPS contained 33 % more fucose but 51 % less glucose than EPS from suspension culture. Glucuronic acid content was influenced by light intensity, being higher at 70 µmol photons $\cdot m^{-2} \cdot s^{-1}$ compared to 330 µmol photons $\cdot m^{-2} \cdot s^{-1}$.

From the whole data and the experiences of TL cultivation of *Netrium digitus* it can be concluded that the TL system can be further considered as a suitable system for EPS production by microalgae. Although the EPS concentration was high in the suspension culture and it can be maintained axenic, this type of cultivation is not feasible for larger applications. Practical restrictions as mixing, light distribution and harvest complicate up-scaling. In contrast, the TL is easily up-scalable and harvest is facilitated by the separation of medium and biomass. Its simple construction also makes the TL-system much less expensive than closed systems.

Knowledge about the handling of EPS producing microalgae on TL, the expected yields under various conditions, the monosaccharide composition and a method for rapid EPS quantification in culture supernatants present a solid framework for future endeavors.

6. Outlook

Exploring new topics in science always leads to more open questions with every step of the road. TL cultivation for EPS production with zygnematophyceaen microalgae is one of those topics. While the possibilities of cultivation and their influences on EPS quantity and quality were assessed, questions arose unremittingly. Starting from the molecular scale, they concern the identity of the unknown monosaccharide and the molecular weight of the one or possibly more polysaccharides constituting the EPS. Especially for cosmetic and nutraceutical applications, this knowledge is of particular interest.

For these applications, further increase in EPS production is also desirable. Combinations of already tested conditions, as high nitrogen and low pH, but also higher light intensities have to be evaluated to maximize yield. Other desmid species should also be tested, since the potential in finding a more productive under the at least 3 000 species (Gerrath, 1993) is immense.

All the previously named improvements are worthless if the biofilm stability stays the limiting factor for the cultivation period. The targeted addition of ions to the medium may pose a solution, but also a change of reactor design towards a horizontally orientated growth area is imaginable. This set-up also bears further advantages in construction simplicity and material use.

Not only at a large production scale, but also in laboratory experiments the influence of other organisms has to be considered. Especially with the production of easy accessible organic carbon in form of EPS, but also for other microalgal applications, close monitoring of heterotrophic contaminations is necessary. With the developing industry of microalgal cultures for biofuels and in particular for products, produced by a specific organism, solutions for contamination control have to be developed. These contaminations include other algae, bacteria, viruses, grazers as well as fungi and may need individual approaches.

For an efficient development of smart and at best eco-friendly solutions, exchange and communication of knowledge are needed. Agriculture can be a good example, where considerable resources are invested into crop protection. In this large and well-established field, it can also be seen that researchers, focusing

on the improvement of the products e.g. increase in yield or improvement of flavor, not necessarily also invest themselves into protective strategies. As the still young field of algal biotechnology will diversify in the coming years and decades, a similar development is likely and also desired. Approaches in crop protection may include mechanical barriers (closed systems, expensive), extreme culturing conditions, which limit growth of unwanted organisms, but also genetically modified microalgae together with matching culturing conditions. Elegant solutions as co-culture with protective organisms have been applied in agriculture (e.g. Stephens et al. (2006)) and analogs may be found for microalgae as the co-culture with antibiotic-producing bacteria.

Another practical question concerns harvesting of larger cultures. Although the harvesting situation in immobilized systems is much improved compared to suspensions, an efficient technique for a technical scale is desirable. As demonstrated in Fig. 3.3a (p. 34) the biofilm displays three sections. The top section is made up of relatively densely packed cells. Below this layer, the cell density is much lower and mostly EPS are present. Attached to the substrate is again a layer of cells. This pre-separation of cells and EPS may be useful for harvesting, especially if the interest is solely on EPS and not on a mixture of EPS and cells. The top layer can be taken off before the EPS layer is removed. The cells attached to the substrate material may then be left on said material and can regrow along with further EPS production.

The possibilities to proceed are plentiful and are not all listed here. It will be interesting to see how the applied field of polysaccharide production by the small but powerful microalgae will develop.

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Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Michael Melkonian betreut worden.

Alice Ekelhof
Köln, den 09. Februar 2017