Interactions between Diatoms and Bacteria from phototrophic Biofilms of the littoral Zone of Lake Constance

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"…we live in the Age of Bacteria.
As it was in the beginning,
is now
and ever shall be,
until the world ends…"

Stephen Jay Gould in:
"The Spread of Excellence from Plato to Darwin"

Contents

General Introduction

Biofilms

"The importance of microorganisms in human health and disease, and the massive impact of the pure-culture approach devised by Robert Koch and others, has understandably led to a philosophy in microbiological research that emphasizes the study of microorganisms in pure liquid culture. This approach has so prominently pervaded microbiology that biofilm research was long neglected until microbiologists "re-discovered" these fascinating communities almost 40 years ago" (Battin et al., 2007).

Exaggerated one could regard the planktonic phase of microorganisms just as a transport mechanism for translocation between surfaces (Watnick & Kolter, 2000) where they can settle to form biofilms. In fact, "most surfaces on this planet teem with microbial life, creating ecosystems of diverse organisms that flourish in slimy beds of their own making" (Kolter & Greenberg, 2006). Biofilms are very complex communities often exhibiting a high phenotypic plurality regarding substrate and nutrient utilization, production of extracellular polymeric substances (EPS) and cell/cell communication, resulting in surprisingly coordinated multicellular behavior, even perceived as "city of microbes" (Watnick & Kolter, 2000). Integrating opportunistic individuals in coordinated units is assumed to be mediated by quorum sensing (Dunlap, 1997; Fuqua et al., 1996; Ruby, 1996). Being an inhabitant of such "biofilm-cities" is often advantageous, e.g. biofilm cells are more resistant against changes in salinity and temperature, UV radiation, desiccation or toxins and detergents (Decho, 2000; Costeron et al., 1987).

Epilithic phototrophic biofilms are based on interactions between the primary producers (algae and cyanobacteria) and bacteria, fungi, protozoa, insects, larvae, shellfish etc. (Makk et al., 2003). For Lake Constance, a large mesotrophic, warm-monomictic lake in central Europe, phototrophic organisms in biofilms on stones, sand, mud and other particles in the littoral zone, are dominated by diatoms (Bahulikar, unpublished data, Fig.1).

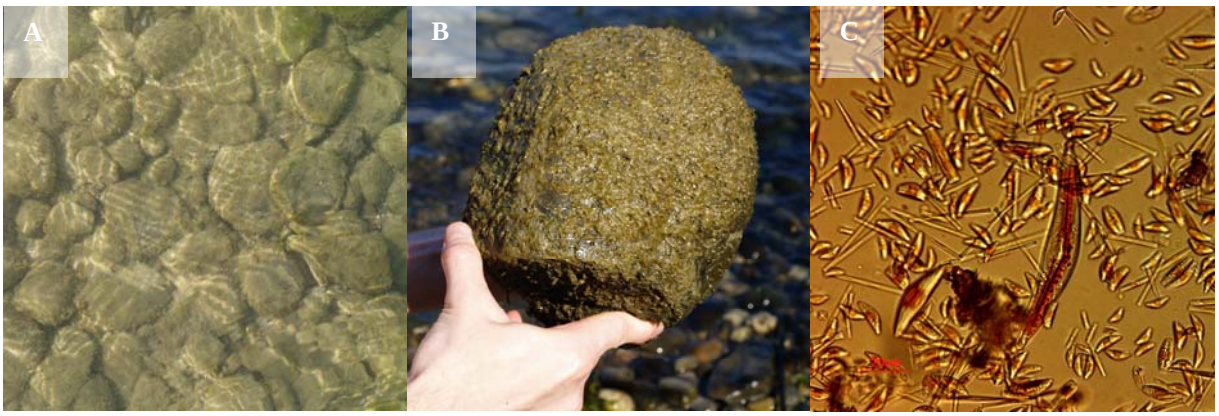

Fig 1: A: Diatom dominated biofilms in the littoral zone of Lake Constance; B: Such biofilms can become several millimeters thick; C: Microscopy reveals a high biodiversity in such biofilms;

Single microbial strains or even individual cells are thought to fill distinct niches within the biofilm, possibly regulated by a complicated “conversation” based on many different soluble signals (Watnick & Kolter, 2000) forming a metacommunity (Battin et al., 2007). In Lake Constance biofilms (Fig.1) are widely distributed, occupying to our observations several square kilometers. These biofilms are exposed to a spatial heterogeneity regarding substrates like rocks, wood, sand, mud, macrophytes, changing water levels, atmospheric pressure, light intensities, nutrient availability etc., thus forming itself an entity that can be regarded as a landscape (Battin et al., 2007).

Besides the living organisms, EPS and trapped water are main components of the biofilms, which are thought to act as a kind of glue sticking particles together, thus stabilizing whole sediments (Stal & Brouwer, 2003; Wigglesworth-Cooksey et al, 2001; Decho, 2000; Sutherland & Grant, 1998).

It is assumed that biofilm formation is initiated by the adsorption of organic and inorganic ions to a surface, followed by the settlement of bacteria which again serve as a substrate for the attachment of eukaryotic algae (Battin et al., 2003). Such an obligatory order for substrate adhesion is discussed controversially (Cooksey & Wigglesworth-Cooksey, 1995).

Diatoms

Diatoms belong to the group of Heterokontophyes (Andersen, 2004) and are classified in two major groups, the mostly radially symmetrical Centrales and the mostly bilaterally symmetrical Pennales (Fig.2). Some diatoms possess combined criteria for Centrales and Pennales and are therefore discussed to belong to a third group (Kooistra et al. 2003). Most centric diatoms are planktonic, while most pennate diatoms are benthic and are associated with solid surfaces.

The chloroplasts of diatoms originated from a secondary endocytobiosis event, where a heterotrophic eukaryotic host cell engulfed a eukaryotic algae (Cavalier-Smith, 2002) and thus may have a metabolism significantly different to higher plants or other algae, whose chloroplasts derived from primary endocytobiosis, where chloroplasts originated from cyanobacteria (Kroth et al., 2008; Wilhelm et al., 2006; Michels et al., 2005). Many diatoms may not depend on photosynthesis, but can live heterotrophic as well (Tuchman et al., 2006; Tan & Johns, 1996; Smayda & Mitchell-Innes, 1974; Lewin, 1953). A conspicuous

morphological feature of diatoms is the cell wall composed of frustules made of silica, consisting of two overlapping parts, the epitheka and the hypotheka. Some structures on these frustules are used for secretion: raphids have one or two grooves in the cell wall, the raphe, which is their main organ for secretion (Fig.2B), araphids (Fig.2A) may secrete polymers via pores in the cell wall, the labiate processes. Pennate diatoms thus can be distinguished into raphid and araphid diatoms (Fig.2). Further classification and details of the frustule are described in Graham & Wilcox (2000).

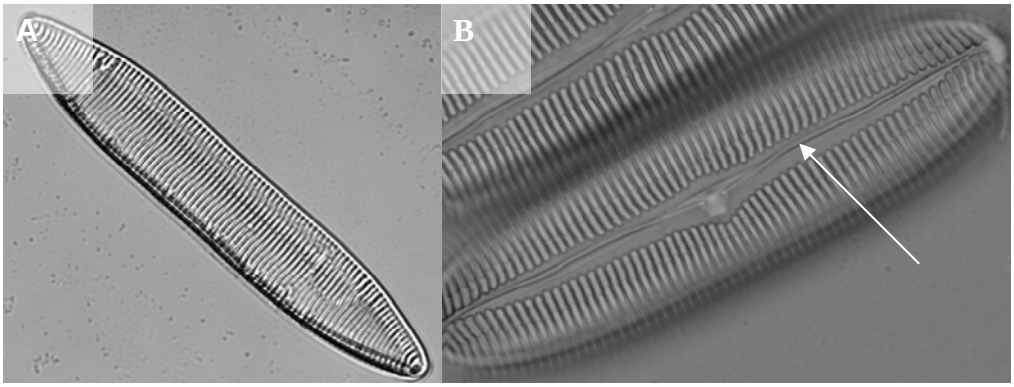

Fig 2: A: Frustules of an araphid *Fragilaria* sp. Lyngbye; B: Frustules of the raphid diatom *Pinnularia viridis* (Nitzsch) Ehrenberg; the raphes are deep grooves in the cell wall as indicated by the arrow;

Axenic Algae

Axenic algae are algae in pure culture without bacteria or any other contaminants. "[…] Bacteria and algae […] are found together in loose or tight associations. Anyone who has tried to grow axenic algal cultures will appreciate the tenacity of some of these associations" (Cole, 1982). First reports about pure algal cultures exist from the late 19th century (Klebs, 1896). In the literature, various methods are described to obtain axenic algae (Conell et al., 1996; Cottrell et al, 1993; Waterbury et al., 1986; Divan & Schnoes, 1982; Guillard, 1973, Hooshaw & Rosowski, 1973, Droop, 1967; Tatewaki & Provasoli, 1964;

Brown & Bischoff, 1962; Machlis, 1962; Spencer, 1952). These methods are based either on physical separation, e.g. ultrasound treatment or differential centrifugation, or on toxin treatment, mostly antibiotics.

Interactions between Diatoms and Bacteria

Most diatom cultures are permanently contaminated by heterotrophic bacteria (xenic culture), even after years of cultivation in the laboratory. Associations of diatoms and intracellular cyanobacteria have been described frequently in literature, often the bacteria profit from carbon sources provided by the diatom, whereas the diatom utilizes nitrogen that is fixed by the endosymbiont (Carpenter & Janson, 2000; Villareal, 1991; Martinez et al., 1983). Some *Pinnularia* sp. Ehrenberg are known to harbor heterotrophic bacteria closely associated with the chloroplast endoplasmatic reticulum (cER). Interestingly the relationship of these organisms is that close, that the cell cycles of bacteria and diatoms are coupled (Schmid, 2003).

Diatoms are mainly associated with extracellular bacteria, usually in close spatial contact. Relevant cross-feeding or chemical interactions between diatoms and bacteria can happen only within a certain distance (excluding gaseous or other highly diffusible substances). Thus a zone around algal cells within which microorganisms are influenced by algal products was defined as “phycosphere”, as an analogy to the rhizosphere of higher plants (Bell & Mitchell, 1972).

Molecular investigations on the community composition of extracellular heterotrophic bacteria associated with diatoms were already performed with samples from different habitats (Grossart et al., 2005; Makk et al., 2003; Schäfer et al., 2002; Riemann et al., 2000; Bowman et al., 1997) as well as with samples from non-axenic (xenic) uni-algal diatom cultures. Such

distinct assemblages of associated bacteria have been termed satellite bacteria (Schäfer et al., 2002; Bell, 1984). For Lake Constance so far only bacteria associated with diatom aggregates in lake snow were investigated. Beside *Alpha-*, *Beta-* and G*ammaproteobacteria*, *Bacteroidetes* were detected (Brachvogel et al., 2001, Weiss et al., 1996), fluctuating in relation to the age of the aggregates (Knoll et al., 2001).

Interaction of phytoplankton and bacteria is assumed to be confined to the degradation and cycling of organic matter produced by the phytoplankton (Grossart et al., 2005; Schäfer et al., 2002). The role of specific classes of bacteria involved in such degradation of organic matter released by diatoms and other algae has been studied in marine (Riemann et al, 2000) and in freshwater systems (Makk et al., 2003; Brachvogel et al., 2001; Riemann & Winding, 2001; Schweitzer et al., 2001; Grossart et al., 1997). It was shown that bacteria can colonize diatoms and degrade diatom derived mucus via ectohydrolases, thus controlling diatom stickiness and aggregation (Azam, 1998).

Co-cultivation experiments of diatoms and extracellular bacteria reveal various effects: bacterial growth and DNA synthesis may be stimulated by diatom exudates (Murray et al., 1986) and vice versa diatom growth may be suppressed or enhanced (Fukami et al., 1997; Hirayama & Hirayama, 1997; Baker & Herson, 1978; Ukeles, R. & Bishop, J., 1975). The adhesion strength of diatoms may be reduced (Wigglesworth-Cooksey & Cooksey, 2005), enhanced (Grossart, 1999), or even fluctuate depending on the cultivation parameters (Gawne et al., 1998). There are indications that extracellular bacterial factors are involved in these effects (Baker & Herson, 1978). Diatom bacteria interactions may be accompanied by a qualitative change in the EPS composition (Grossart, 1999; Wigglesworth-Cooksey & Cooksey, 2005) and can be regarded as a key factor for aggregate formation (Grossart et al., 2006).

Only a few studies were done to investigate algae-bacteria interactions on a functional genetic level or to find extracellular factors that are exchanged between these organisms.

Molecular analyses and cultivation approaches show, that many algae cannot synthesize vitamin B12 and thus may get this co-enzyme from bacteria (Croft et al, 2005; Cole, 1982).

Extracellular polymeric Substances (EPS)

"EPS is an operational definition designed to encompass a range of large microbially-secreted molecules having widely varying physical and chemical properties, and a range of biological roles" (Decho, 2000).

Diatom EPS (Fig.3) mainly consists of polysaccharides and proteins (Chiovitti et al., 2003), bacterial EPS may consist of polysaccharides, proteins and nucleic acids. Parts of the EPS are soluble, other parts are colloidal to solid. The polymer chemistry and the surface properties of EPS are thought to play an important role for aggregate formation (Bhaskar et al., 2005), water congestion (Potts, 1994) or as ion trap (Chin et al., 1998). Even pathways for fixation of inorganic carbon are discussed to be localized extracellular (Puscaric & Mortain-Bertrand, 2003).

Diatoms may secrete EPS for different reasons. Some raphid diatoms secrete polysaccharides and glycoproteins for cellular movement on substrates (Graham & Wilcox, 2000; Pickett-Heaps, 1991), other diatoms secrete pseudo filamentous tubes or capsules (Fig.3A), while again other diatoms use EPS to attach to substrates, or for the formation of cell aggregates, capsules, stalks, etc. (Hoagland et al., 1993). For attachment the quality of EPS is more important than the quantity (Becker, 1995). Diatom attachment is thought to be an active process that requires glycoproteins and metabolic energy (Dugdale et al., 2006; Chiovitti et al., 2003, Cooksey & Wigglesworth-Cooksey, 1995). In mixed biofilm communities diatom EPS might interact specifically with bacterial EPS by forming colloidal structures (Gawne at al., 1998).

Extracellular polysaccharides from diatoms consists mainly of the monomers rhamnose, fucose, xylose, mannose, galactose and glucose, whereas glucose and galactose are often described as the dominant entities in uni-algal cultures (Bhaskar et al., 2005; Underwood et al., 2004; Chiovitti et al., 2003; Staats et al., 1999) as well as in whole natural biofilm communities (Battin et al., 2003; Taylor et al. 1999). It is assumed that the EPS may be used by heterotrophic organisms as a carbon source. First studies indicate a selective degradation of diatom derived polysaccharides by heterotrophic bacteria (Giroldo et al., 2003). It was shown that diatom derived carbohydrates affect the community composition of associated bacteria (Haynes et al., 2007).

Polysaccharide secretion by diatoms may depend on varios factors. The influence of nutrient availability is already well studied. Phosphate limitation or salinity changes e.g. increase the polysaccharide production by *Phaeodactylum tricornutum* Bohlin cultures and cause an accumulation of deoxy- and O-methylated sugar monomers, thus enhancing the hydrophobilcity of the polysaccharides (Abdullahi et al., 2006). *Achnanthes brevipes* C. Aqardh (Guerrini et al, 2000) and *Cylindrotheca fusiformis* Reimann & Lewin (Magaletti et al., 2004) react to phosphate limitation with enhanced polysaccharide secretion as well. Comparable high concentrations of ammonium sulfate, ammonium nitrate or urea lead to increased EPS secretion in *Phaeodactylum tricornutum* cultures (Guzmán-Morillo et al., 2007). Underwood et al., 2004, demonstrated enhanced EPS secretion at different nutrient limiting conditions for various diatoms. Moreover diatom EPS secretion seems to be regulated by the diurnal rhythm (Tuchmann et al., 2006; Orvain et al., 2003, Smith & Underwood, 2000).

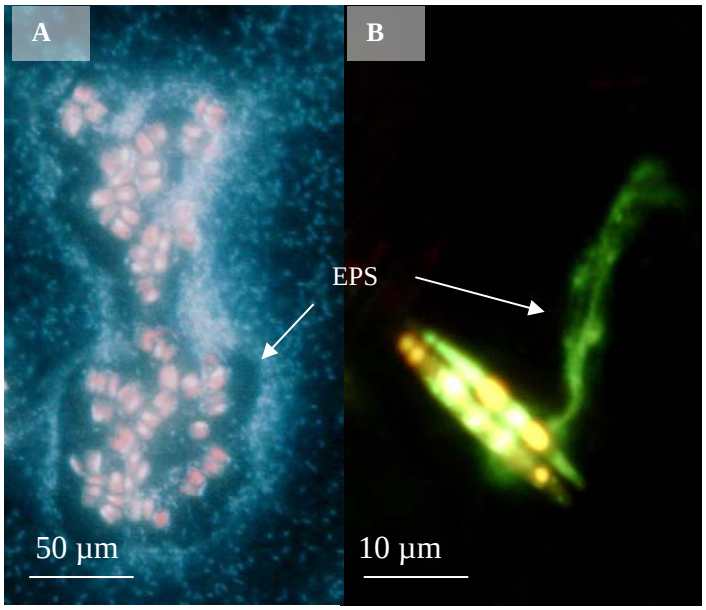

Fig 3: A: Epi-fluorescence micrograph of a DAPI-preparation of a *Cymbella microcephala* Grunow biofilm including associated bacteria. The diatom aggregates (red) are surrounded by an EPS matrix keeping bacteria (blue) at bay. B: Epi-fluorescence micrograph of a SybrGreen-preparation of an unknown diatom. This species seems to secrete nucleic acids as EPS.

Overview

In this study we developed methods to purify diatoms systematically from associated bacteria. We found that most diatoms do not produce biofilms any more when axenic. Therefore the interaction between diatoms and bacteria is thought to be a key element in such biofilm formation.

We mapped the bacterial community composition of bacteria associated with single diatom strains via 16S rRNA-gene clone libraries and performed defined diatom-bacteria co-cultures to monitor bacterial influences on diatom growth and EPS secretion. Phylogenetic studies on bacterial 16S rRNA-genes and bacterial utilization of diatom polysaccharides indicate that *Proteobacteria* and *Bacteroidetes* adapted to micro-niches in diatom biofilms.

Growth of most diatoms is strongly influenced by heterotrophic bacteria. Thus the interaction with bacteria can be regarded as an important factor contributing to the succession

of certain diatom strains in the natural environment. By correlating concentrations of free dissolved amino acids (DFAA) with diatom growth in diatom/bacteria co-cultures, we hypothesize, that DFAA may be either involved in regulating diatom growth, or that bacteria may influence DFAA release by the diatoms.

Heterotrophic bacteria also influenced EPS secretion of most diatoms. Polysaccharide secretion was influenced mainly quantitatively, while protein secretion was influenced quantitatively and qualitatively. By performing meta-proteomic experiments regarding diatom/bacteria interactions we characterized extracellular proteins that are induced during such biofilm formation. Database analysis indicates characteristic functions of these proteins within diatom-bacteria biofilms.

Protocols for the Removal of Bacteria from benthic Diatom Cultures

Chapter 1

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Key words: axenic cultures, bacteria, benthic, biofilm, diatom, purification

Abbreviations: CTAB, Cetyltrimethylammoniumbromid**;** DAPI, 4',6-Diamidino-2-phenylindol; DM, diatom medium; EPS, extracellular polymeric substances; PBS, phosphate buffer saline

Abstract

In this report we describe different combinations of physical separation and antibiotic treatment to remove associated bacteria from freshwater diatoms. Diatoms were purified either from natural epilithic biofilms or from uni-algal cultures. We found that for most strains different purification procedures have to be combined individually. In a new approach we show that for some diatom strains the substitution of associated aquatic bacteria by an antibiotic sensitive *E. coli* strain and subsequent treatment with antibiotics may be a successful approach to obtain axenic diatom cultures.

Introduction

Diatoms are generally found to be associated with heterotrophic bacteria, especially *Alphaproteobacteria* and bacteria from the *Bacteroidetes* group, both, in nature as well as in most stock cultures (Grossart et al. 2005; Makk et al. 2003; Schäfer et al. 2002; Brachvogel et al. 2001; Knoll et al. 2001; Riemann et al. 2000; Bowmann et al. 1997; Weiss et al. 1996). Modern molecular techniques (Kroth, 2007) and analyses of diatom/bacteria interactions and of EPS production (Staats et al. 1999) demand for axenic diatom cultures. Generally cultures containing a single algal strain are referred to as uni-algal cultures, while bacteria-free uni-algal cultures are termed axenic. First reports on axenic algae exist from the late 19th century (Klebs, 1896). Various procedures for purification of algae in general are described in the literature (Conell and Cattolico, 1996; Cottrell and Suttle, 1993; Waterbury et al. 1986; Divan and Schnoes, 1982; Guillard, 1973; Hooshaw and Rosowski, 1973; Droop, 1967; Tatewaki and Provasoli, 1964; Brown and Bischoff, 1962; Machlis, 1962; Spencer, 1952); however, in some cases they are not always repeatable or not suitable for different species. Benthic

diatoms often are difficult to purify due to the large amount of sticky mucilage produced by the algae, making it difficult to remove bacteria living within this mucilage. We have tested various procedures for purification of diatoms including various antibiotic treatments, additions of supplementary substances, heat treatment, different physical separations, and defined co-cultures. Most of them were not resulting in viable axenic diatoms. Here we present five successful approaches for the purification of benthic diatoms by combinations of different methods: physical separation via ultrasonic treatment, vortexing, filtration, differential centrifugation, treatment with various antibiotics and replacement of diatom associated bacteria by *E. coli* cells combined with subsequent antibiotic treatment.

Methods to verify the axenic State of a Culture. To check for bacterial contamination we applied various methods: (i) Phase contrast microscopy or differential interference contrast microscopy (DIC) were utilised as a first estimate of the degree of bacterial contamination; however, only abundant bacterial contaminations were detected this way. Better detection efficiency was obtained by (ii) plating cultures on nutrient rich agar plates, as well as by (iii) DNA staining in combination with epi-fluorescence microscopy using 1000-fold magnification. For the latter approach 0.1 µl SYBR Green I solution was added to 100 µl of suspended culture. After incubation for 10 min, the cells were observed under a microscope. For DAPI staining 30-50 µl of the cultures were dried on a 10-well microscopy slide (Menzel-Gläser, Braunschweig). 20 µl of a 2 $\mu g \cdot ml^{-1}$ DAPI solution in water or PBS was added to every well. After five minutes of incubation the whole slide was rinsed first with water and then with ethanol. The cells were dried again and stabilized in AF1 (Citifluor, London). We genererally preferred SYBR Green instead of DAPI staining because of the stronger fluorescence and thus sensitivity and the easier handling.

We checked the reliability of the methods described above by attempts to amplify bacterial 16S rRNA genes from a purified *Synedra ulna* (Nitzsch) Ehrenberg strain. For DNA

preparations the diatoms were pelleted by centrifugation and frozen in liquid nitrogen, then crushed with a pestle, followed by 1 h incubation at 65°C in 500 µl CTAB extraction buffer (Murray and Thompson, 1980). After cooling to room temperature, the sample was washed with a chloroform-isoamylalcohol mixture 24:1 (v/v). DNA was precipitated with 0.7 volumes isopropanol, washed with 80% ethanol and dried. The dried DNA pellets were dissolved in autoclaved Millipore water (modified after Murray and Thompson, 1980). The purified DNA was used to amplify 16S rRNA genes using the universal bacterial primers 27f 5'-AGA GTT TGA TCC TGG CTC AG-3' (Edwards et al. 1989) and 1492r 5'-TAC GGY TAC CTT ACG ACT T-3' (Weisburg et al. 1991). The generation of amplified DNA products were checked on 1% agarose gels, purified using the NucleoSpin kit (Macherey-Nagel, Düren) and cloned in "*E. coli* XL1 blue excel" using the pGEM-T cloning kit (Promega, Leiden). 150 randomly selected colonies were screened via restriction digest using the enzyme MspI (Fermentas, Burlington) according to the manufacturer's instruction. The resulting fragments were analyzed via agarose gel electrophoresis (2%). These restriction patterns were compared and fragments related to unique patterns were sequenced. All screened clones obtained from cultures that previously had been declared to be axenic by SYBR Green staining and plating on nutrient agar contained 16S rRNA sequences all originating from chloroplasts and mitochondria of the diatom, but not from bacteria (data not shown). Xenic cultures served as template for positive control reactions.

Generation of axenic Diatom Strains directly from natural Biofilms. In two approaches the diatoms were purified directly from environmental biofilms (Fig.1, paths A and B; Tab.1). Stones with attached biofilms were taken from the littoral zone of Lake Constance, Germany. The biofilms were scraped off, transferred to a centrifugation tube and suspended in liquid Diatom Medium (DM) (Watanabe, 2005). This mixture was vortexed for 10 min or treated for 10 sec with ultrasound (ultrasonic processor UP50, Dr. Hielscher GmbH) with an amplitude

of 40% at a frequency of 0.5 sec (Brown and Bischoff, 1962). From here we followed two strategies: In the first approach (Fig.1, path B), either 50 or 100 µl were spread or streaked on DM agar plates, containing either 10 $\mu g \cdot ml^{-1}$ kanamycin and tetracycline, 50 $\mu g \cdot ml^{-1}$ ampicillin, or a combination of 270 $\mu g \cdot ml^{-1}$ penicillin G, 135 $\mu g \cdot ml^{-1}$ streptomycin and 27 $\mu g \cdot ml^{-1}$ chloramphenicol (according to Guillard, 1973). In addition we used antibiotic-free DM plates. The plates were incubated for three days in a 16:8 h light cycle at 16°C and 30 - 60 µE using 58 W TLD PHILLIPS neon lamps (standard conditions for all diatom strains). Using an inverse microscope, single diatom cells were marked on the bottom part of the petri dishes and transferred to suspension plates filled with 1 ml DM using a preparation needle. The agar plates were incubated further until algal colonies appeared. Cells from the resulting colonies were also cultured on suspension plates. Small diatoms of 2-3 µm length were streaked repeatedly to isolate different species before cells were transferred to liquid DM. Growing cultures were checked for bacterial contaminants via epi-fluorescence microscopy after SYBR Green I (Invitrogen, Paisley) staining. Axenic strains then were transferred to 100 ml Erlenmeyer flasks. With this approach we were able to purify 77 diatom strains from biofilm samples representing approximately 40 different species as determined by light microscopical analyses. The cell size of most strains was smaller than 20 µm. Species larger than 100 µm did not survive, except *Synedra ulna*. In another approach (Fig.1, path A) we filtered suspended biofilm through a filter of two-micrometer pore size according to Waterbury et al. (1986). The remaining particles on the filter surface were resuspended in DM and filtered again. This process was repeated three times. Small parts of the remaining residue in the filter were picked up randomly with an inoculation loop and streaked on agar plates as described above. Cells from resulting colonies were transferred to liquid medium as well as to DM agar plates as described. Interestingly, this approach yielded various axenic colonies of unknown fast growing algae smaller than five micrometers, which were axenic, whether they were inoculated with or without antibiotics (Fig.1/Tab.1). This phenomenon can be explained

by the faster growth of these algae, generating colonies within four days, while the aquatic bacteria developed a dense layer only after six to nine days.

Table 1: Summary of the successfully used approaches to separate diatoms and bacteria showing separation techniques and further treatments;

source	separation	first antibiotics	second antibiotics	third antibiotics	fourth antibiotics	other treatments	axenic diatoms
biofilms	vortexing	kanamycin tetracycline	-	-	-	-	2 unidentified strains
biofilms	vortexing	penicillin G streptomycin chloramphenicol	-	-	-	separation (dilution/ streaking) on medium	11 unidentified strains
biofilms	vortexing or ultrasound	penicillin G streptomycin chloramphenicol	-	-	-	-	63 unidentified strains
biofilms	vortexing	ampicillin	-	-	-	-	1 unidentified strain
biofilms	vortexing or filtration	-	-	-	-	streaking on medium	various small unidentified strains
biofilms	vortexing or filtration	kanamycin tetracycline	-	-	-	streaking on medium	various small unidentified strains
biofilms	vortexing or filtration	penicillin G streptomycin chloramphenicol	-	-	-	streaking on medium	various small unidentified strains
biofilms	vortexing or filtration	ampicillin	-	-	-	streaking on medium	various small unidentified strains
xenic cultures	centrifugation	penicillin G streptomycin chloramphenicol	-	-	-	dissolution of EPS	*Cymbella microcephala* *Synedra acus* var. *angustissima* *Fragilaria pinnata* three strains of *Synedra ulna*
xenic cultures	ultrasound	penicillin G streptomycin chloramphenicol	-	-	-	-	*D164*
xenic cultures	ultrasound	penicillin G streptomycin chloramphenicol	kanamycin tetracycline	-	-	-	*Achnanthes lineaus* *Gomphonema clavatum*
xenic cultures	ultrasound	penicillin G streptomycin chloramphenicol	tetracycline	ampicillin	-	co-culture with *E. coli*	*Navicula cincta*
xenic cultures	ultrasound	penicillin G streptomycin chloramphenicol	tetracycline	ampicillin	kanamycin tetracycline	co-culture with *E. coli*	

Generation of axenic Diatom Strains from xenic Cultures: Ultrasound, heavy Antibiotic Treatment and "single Cell Care". Xenic diatom cultures of *Achnanthes linearis* (W. Smith) Grunow, *Achnanthes* sp. Bory, *Caloneis silicula* (Ehrenberg) Cleve, *Cyclotella meneghiniana* Kützing, *Fragilaria pinnata* Ehrenberg, *Gomphonema clavatum* Ehrenberg, *Gomphonema* sp. Ehrenberg, *Navicula capitatoradiata* Germain, *Navicula cincta* (Ehrenberg) Kützing, *Nitzschia dissipata* (Kützing) Grunow, *Nitzschia dissipata* var. *media* (Hantzsch.)

Grunow, *Nitzschia palea* var. *debilis* (Kützing) Grunov, *Nitzschia sigmoidea* (Ehrenberg) W. Smith *Pinnularia viridis* (Nitzsch) Ehrenberg, *Staurosira* sp. Ehrenberg, *Synedra acus* var. *angustissima* (Grunov) van Heurck, and *Synedra ulna* were treated with ultrasound as described (Fig.1, path C). These diatoms are difficult to purify because of strong EPS production which results in clumping cell aggregates. Separation efficiency and intactness of the diatom cells were analyzed by phase contrast microscopy. Ultrasound treatment as described above led to a non-clumping cell suspension with intact diatom cells for most strains. Between 10 µl and 200 µl of the suspended cells were spread on DM agar plates containing 170 $\mu g \cdot ml^{-1}$ penicillin G, 85 $\mu g \cdot ml^{-1}$ streptomycin and 17 $\mu g \cdot ml^{-1}$ chloramphenicol. After three days of incubation, single cells were marked and transferred to liquid DM as described above. These cultures were checked daily by inverse microscopy. After eleven days, growing cultures were screened for bacterial contaminations as described above. Axenic cultures were transferred to new suspension plates. Still contaminated cultures were streaked on DM agar plates containing tetracycline and kanamycin (10 $\mu g \cdot ml^{-1}$ each) and treated as described above. Axenic cultures with reasonable growth rates were transferred from the suspension plates to 100 ml Erlenmeyer flasks containing liquid DM. This approach worked well for three different strains of *Synedra ulna*, one strain of *Fragilaria pinnata* and an unidentified strain (D164).

Generation of axenic Diatom Strains from xenic Cultures: Antibiotic Treatment and Co-Cultivation with Escherichia coli. Some diatom strains contained associated bacteria which had such a strong resistance, that treatments with rising antibiotic concentrations resulted in the death of the diatoms before the bacteria were affected effectively. We found that many diatoms in co-culture with bacteria grew denser and faster than while being axenic. Often such bacterial effects on diatom growth were inducible by *E. coli*. Thus we tried to substitute aquatic bacteria in diatom cultures by *E. coli* (Fig.1, path D; Tab.1). The diatom

strains of *Achnanthes linearis*, *Achnanthes* sp., *Asterionella ralfsii* W. Smith, *Gomphonema clavatum*, *Gomphonema* sp., *Navicula cincta*, *Nitzschia palea* var. *debilis*, *Punctastriata* sp. Williams and Round, and *Synedra acus* var. *angustissima* were treated with ultrasound as described before. First we tried to incubate the xenic diatom cells directly with the *E. coli* strain XL1 blue (being resistant to tetracycline and sensitive to ampicillin; Stratagene, La Yolla) on plates as well as in liquid culture, resulting in a strong growth of the diatom associated biofilm bacteria, while the diatoms did not grow or were simply overgrown by the bacteria. Therefore we followed another strategy based on the hypothesis that antibiotic treatment at high concentrations may weaken the diatom associated bacteria. The critical factor for this approach is to choose an incubation period with the antibiotics that weakens the bacteria but does not kill the diatoms. Diatom cultures, subsequently treated for a short period with strong antibiotics followed by addition of *E. coli* bacteria and fresh medium, then show a substitution of the diatom associated bacteria by *E. coli*, possibly because of its faster growth. This procedure is supported by a mild tetracycline treatment. As the *E. coli* strain is sensitive to ampicillin, in a next step the *E. coli* cells can be removed by addition of the respective antibiotic in low concentrations. Best results were obtained when 50-100 µl xenic cell suspensions were spread on DM agar containing 170 $\mu g \cdot ml^{-1}$ penicillin G, 85 $\mu g \cdot ml^{-1}$ streptomycin and 17 $\mu g \cdot ml^{-1}$ chloramphenicol and incubated for one day. The diatom cells then were marked, excised as described and transferred to suspension plates. These suspension plates contained 1 ml DM medium and 3 µl of a dense over night culture of *E. coli* per well. Cultures were checked daily by inverse microscopy. Dense cultures were suspended by 1 min vortexing and streaked on DM agar plates (0.25 $\mu g \cdot ml^{-1}$ tetracycline) to select for tetracycline resistant diatom/*E. coli* consortia, expecting other bacteria to be weakened by the antibiotic. After three days of incubation, diatom cells were excised as described and transferred to fresh DM medium in suspension plates. Dividing cells were then streaked on DM plates containing 50 $\mu g \cdot ml^{-1}$ ampicillin. In parallel, the cultures of diatom/*E. coli*

consortia on DM agar plates containing tetracycline were incubated additionally further and screened for developing algal colonies. In case visible diatom colonies appeared, these diatom/*E. coli* associations were removed and treated with ampicillin as described above. After 20 days of incubation on ampicillin again single diatoms were marked and excised as described and transferred to suspension plates with fresh DM. In addition, the agar plates were kept and observed for diatom colonies. Cells from colonies were transferred to 1 ml of fresh DM. The cultures in the suspension plates were screened for bacterial contaminants as described above and axenic strains were transferred to 100 ml Erlenmeyer flasks. Diatoms with weak bacterial contaminations were inoculated again in liquid DM containing 2.5 $\mu g \cdot ml^{-1}$ kanamycin and tetracycline. After a short growth period, the cells were transferred to liquid DM. By this approach we were able to purify *Achnanthes linearis*, *Gomphonema clavatum* and *Navicula cincta*.

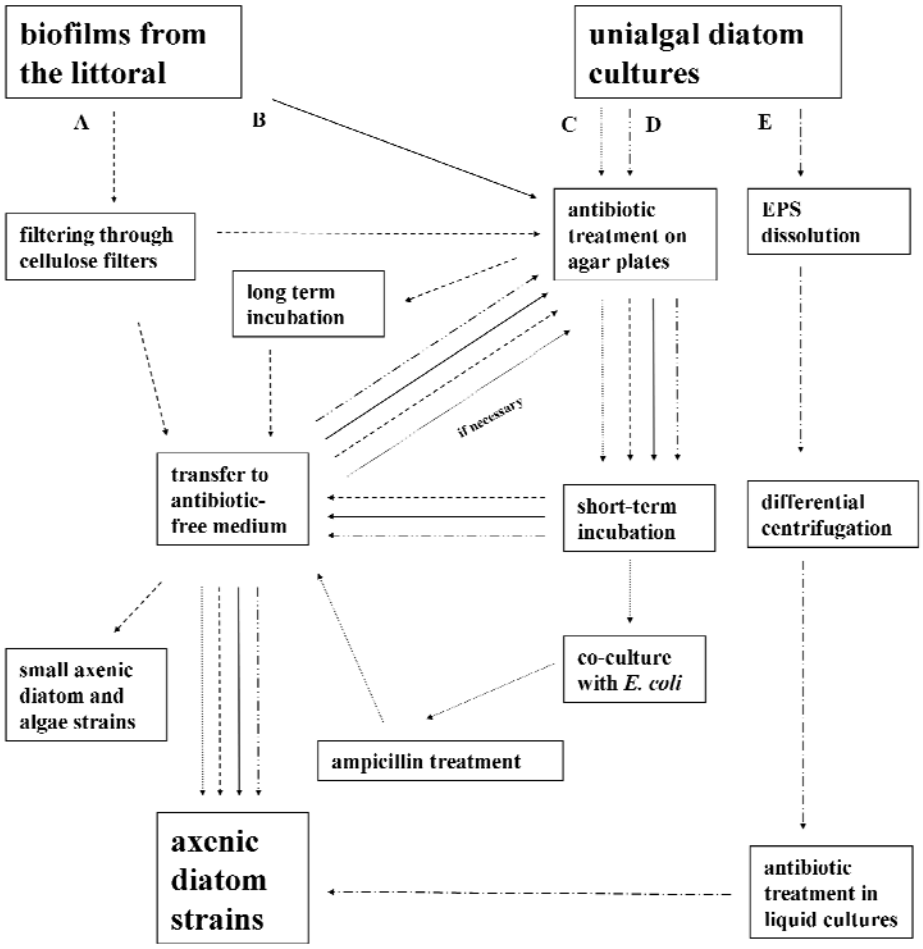

Figure 1: An overview of key steps that were performed during the purification of different benthic freshwater diatom strains. The different arrows represent the four successfully used strategies (A-D). (A, B) Approaches for isolation from environmental samples, (A) separation of diatoms and bacteria by filtration as the initial step, (B) isolation of diatoms after initial antibiotic treatment, (C,D) approaches for isolation from unialgal xenic cultures (C) short term antibiotic treatment, (D) co-cultures with *E. coli* as intermediate step to obtain axenic diatoms. See text for a detailed discussion of the individual procedures.

Generation of axenic Diatom Strains from xenic Cultures: Dissolution of EPS, differential Centrifugation and moderate Antibiotic Treatment. Another possibility to separate bacteria/diatom aggregates is the dissolution of frustule associated EPS. Here 1 ml of culture was heated to 30°C for two hours under gentle shaking conditions (Staats et al. 1999), followed by 1 minute vigorous shaking (vortexing) and a centrifugation for one minute at 200 *g* to spin down the diatoms. The supernatant was removed carefully by pipetting and the

pellet was resuspended in 1 ml of DM. Vortexing, centrifugation and resuspension in fresh medium were repeated six times according to Hooshaw and Rosowski (1973), then the cells were resuspended in 100 µl DM. 5 µl aliquots were transferred to suspension plates filled with DM with or without a mixture of 17 $\mu g \cdot ml^{-1}$ penicillin G, 8.5 $\mu g \cdot ml^{-1}$ streptomycin and 1.7 $\mu g \cdot ml^{-1}$ chloramphenicol. In some samples also a small amount of lysozyme was added. The use of a mixture of penicillin G, streptomycin and chloramphenicol in the mentioned low concentrations, combined with the removal of frustule associated EPS, vortexing and differential centrifugation, yielded two axenic diatom cultures: *Synedra acus* var. *angustissima* and *Cymbella microcephala* Grunow (Fig.1, path E). Overall, this method turned out to be very laborious, especially to find the right conditions for differential centrifugation and it was only suitable for a small number of diatoms strains.

Summary and Discussion

Purification of diatoms by removal of bacterial contaminants is essential for various experiments that require axenic diatom strains. According to our analyses, for smaller fast growing diatoms (1-5 µm) streaking on agar plates is often sufficient to separate the organisms without the need of antibiotics. For larger benthic diatoms it may be helpful to observe uni-algal but xenic diatom cultures microscopically to estimate the relationship between the cell numbers and the cell sizes of diatoms and bacteria. Depending on this relationship, cultures should be treated individually. We found it useful to start the purification process during the exponential growth phase of the diatom cultures, when the number of bacteria was comparably low. Another important aspect is the physical removal of bacteria from bound diatom EPS by ultrasound or vortexing. The frequency and duration of both treatments have to be defined individually for each culture. Best results were obtained by

spreading the diatoms after ultrasound treatment on agar plates containing high concentrations of antibiotics followed by removal of single cells just before the diatom cells started to die. Typical indications for cell death were bleaching (in most strains), vesiculation of the cytoplasma (*Synedra sp.* Ehrenberg, *Fragilaria sp.* Lyngbye) or circular movements (motile diatoms like *Pinnularia sp.* Ehrenberg). The incubation on antibiotic plates often had to be repeated, however, it was helpful to include a recovery phase on antibiotic free medium for one to four weeks. Three diatom cultures were purified by substituting the associated bacteria with *Escherichia coli*, a method that was particularly helpful when all other approaches failed including mechanical or antibiotic treatment.

Purification of diatoms from uni-algal cultures usually was more difficult and less successful than from biofilm samples. We found the combination of the antibiotics penicillin G, streptomycin and chloramphenicol in a ratio of 10:5:1 to be the most successful treatment to remove bacteria. Generally higher concentrations of antibiotics combined with short-term incubations were more efficient than using low concentrations for longer periods.

Most diatoms in this study could not be cultivated in an axenic state over a long term. On average our axenic cultures only survived for 1.5 years when sub-cultured monthly. Several of the strains began to reduce the cell size rapidly, while others suddenly formed clumping, slow growing cell aggregates or did not grow after inoculation in new media. Depending on the diatom strain such effects were observed after two to 18 months of cultivation in axenic conditions. Xenic cultures of the same strains on the other hand often survived much longer and could be used repeatedly for purification. Apparently many biofilm diatoms need substances of bacterial origin, indicating a strong cross-feeding interaction as known from other multi species biofilms (Ward et al., 1998). In some cases vitamins like B12 possibly secreted by bacteria have been identified to be important for diatom growth (Croft et al. 2005), however, our culture media were supplemented with Vitamin B12, Biotin and Thiamine.

In the literature we found different methods to verify the purity of an algal culture (Bhaskar et al, 2005; Grossart et al. 2005; Wigglesworth-Cooksey and Cooksey, 2005; Underwood et al. 2004, Chiovitti et al. 2003; Wigglesworth-Cooksey et al. 2001; Baker and Herson, 1978). In some cases the absence of bacteria was demonstrated by phase contrast microscopy which requires vast experience because slight contaminations may be overlooked. Others checked diatom strains by plating the cells on nutrient agar, while Divan and Schnoes (1982) inoculated samples in nutrient rich liquid media, observing for resulting turbidity by bacterial growth. We found the use of nutrient media often to be useful, but we noticed that some bacteria in diatom cultures did not grow in these media. Given the costs and work needed for 16S rRNA-gene amplification and sequencing, we found epi-fluorescence microscopy with SYBR Green stained samples to be the most powerful tool to proof the absence of bacteria.

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Bacteria associated with benthic Diatoms from Lake Constance: Phylogeny and Influences on Diatom Growth and EPS Secretion

Chapter 2

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Abstract

The composition of diatom-associated bacterial communities was studied with 14 different uni-algal xenic diatom cultures isolated from freshwater epilithic biofilms of Lake Constance, Germany. A clear dominance of *Alphaproteobacteria* was observed, followed by *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes* and *Verrucomicrobia*. Pure cultures of the diatom *Cymbella microcephala*, which was found to be dominant in epilithic biofilms in Lake Constance, were co-cultivated with six associated bacterial strains. All these bacterial strains were able to grow in *C. microcephala* cultures in the absence of organic co-substrates. Diatom growth was generally enhanced in the presence of bacteria, and polysaccharide secretion was generally increased in the presence of *Proteobacteria*. The monomer composition of extracellular polysaccharides of *C. microcephala* changed in relation to the presence of different bacteria, but the dominant monomers were less affected. Our results indicate that these changes were caused by the diatom itself rather than by specific bacterial degradation. One *Bacteroidetes* strain strongly influenced carbohydrate secretion by the alga via extracellular soluble compounds. Biofilms were formed only in the presence of bacteria. Phylogenetic analysis and co-culture studies indicate an adaptation of *Proteobacteria* and *Bacteroidetes* to the micro-environment created by the diatom biofilm.

Introduction

Lake Constance is a large mesotrophic freshwater lake in Central Europe. The littoral zone of this lake is an area of high primary production. Rocks in these shallow-water zones are covered by greenish or brownish biofilms, sometimes several millimeters thick, consisting mainly of algae and bacteria. Interaction of algae and bacteria is assumed to be confined to the degradation and cycling of organic matter produced by the algae (Grossart et al., 2005; Schäfer et al., 2002). The role of specific classes of bacteria involved in such degradation of organic matter released by diatoms and other algae has been studied in marine (Riemann et al., 2000) and in freshwater systems (Makk et al., 2003; Brachvogel et al., 2001; Riemann & Winding, 2001; Schweitzer et al., 2001; Grossart et al., 1997). Epilithic biofilms are complex communities, based on interactions between the primary producers (algae and cyanobacteria) and bacteria, fungi, protozoa, insects, larvae, shellfish etc. (Makk et al., 2003). Xenic uni-algal diatom cultures also harbor a distinct assemblage of associated bacteria which have been termed satellite bacteria (Schäfer et al., 2002; Bell, 1984). Usually such algal cultures are maintained for several years and thus the associated bacteria undergo selection. The literature reports on *Proteobacteria* and *Bacteroidetes* as the major bacterial partners found in diatom mats and in diatom cultures from worldwide sampling places and different habitats (Grossart et al., 2005; Makk et al., 2003; Schäfer et al., 2002; Brachvogel et al., 2001; Knoll et al., 2001; Riemann et al., 2000; Bowman et al., 1997; Weiss et al., 1996). As diatoms are the major primary producers and early colonizers of surfaces (Ács, 1998), studies on associated bacteria, in uni-algal cultures and co-cultures of axenic diatoms and bacteria, could help to understand the role of these organisms in natural biofilm formation.

Biofilms are stabilized by extracellular polymeric substances (EPS). Parts of the EPS are soluble whereas other parts are associated with the cell or the substratum, forming jelly-

like to solid structures termed bound EPS. The extracellular polysaccharides of benthic diatoms are commonly composed of rhamnose, fucose, xylose, mannose, galactose, glucose, and other monomers; galactose and glucose often form the major part (Underwood et al., 2004; Chiovitti et al., 2003; Staats et al., 1999; Bowman et al., 1997). These sugar monomers are also found in natural biofilms dominated by diatoms (Battin et al., 2003; Shewan & Mc Meekin, 1983). Such extracellular polysaccharides may be used by heterotrophic organisms as a carbon source (Grossart & Simon, 2007; Haynes et al., 2007; Grossart et al., 2006; Giroldo et al., 2003). In fact, many diatom cultures are permanently contaminated with bacteria, although most diatom culture media do not contain appreciable amounts of organic compounds. Therefore cross feeding between the auto- and heterotrophs is very likely, revealing a strong interdependency which is thought to be one of the major forces of microbial co-evolution of mixed biofilm communities (Ward et al., 1998). Grossart et al., 2005, reported on fluctuations of various bacteria during the different growth phases of diatom cultures. Until now, diatoms and bacteria have rarely been cultivated in defined co-cultures. The observed effects were often ambiguous. Growth of diatoms can be enhanced or can be suppressed by bacteria (Grossart & Simon, 2007; Grossart et al., 2006; Baker & Herson, 1978); also the strength of adhesion to a substratum can be increased (Grossart, 1999) or reduced (Wigglesworth-Cooksey & Cooksey, 2005) or even can fluctuate, depending on the growth conditions of the associated bacteria (Gawne et al., 1998). This is accompanied by changes within the diatom EPS (Grossart, 1999), as indicated by lectin labeling (Wigglesworth-Cooksey & Cooksey, 2005). Thus, diatom/bacteria interactions may vary from being symbiotic to being antagonistic. For most diatoms it is unknown yet whether they actually benefit from bacteria. Heterotrophic bacteria need organic matter for their energy metabolism, but it is still unclear what they feed on within the micro-environment diatom

biofilm and whether they occupy specific niches. For instance *Vibrio proteolytica* is known to feed on exudates from *Amphora coffaeformis* (Murray et al., 1986).

In the present study we address the relation between diatom polysaccharide secretions and associated heterotrophic bacteria. We demonstrate that extracellular diatom polysaccharides can be substrates for these bacteria and that different bacterial strains utilize different parts of the polysaccharides. Inter-species relations were identified by mapping the community structure of diatom-associated bacteria in uni-algal diatom cultures, and by co-cultivation of a representative diatom with the respective bacteria under defined conditions.

Materials and Methods

Diatom Cultures. For isolation and cultivation of diatoms, 5-10 µl of biofilm material was scraped from stones collected in the littoral zone of Lake Constance, suspended in 1.0 - 1.5 ml of sterile diatom medium (DM) (Watanabe, 2005) and homogenized. Single diatom cells were isolated and cultured in DM. The cultivation parameters were 16° C permanently during a 16 h light period and eight hours darkness. The light intensity varied from 30 to 60 µmol photons m^{-2} sec^{-1} using 58 W TLD PHILIPS neon lamps. Cells were sub-cultured monthly and maintained in the same medium.

Isolation and Cultivation of C. microcephala *and bacterial Isolates. C. microcephala* was isolated and cultivated for more than two years as described above. This uni-algal xenic culture was vortexed and diluted in 1:10 steps in DM. From the 10^5 to 10^7 dilution, 50 µl each was spread on 1.5% agar plates with different media: “nutrient broth soyotone yeast extract (NSY) medium” (modified after Hahn, 2003), containing 0.1 $g \cdot l^{-1}$ peptone, yeast-extract and nutrient broth, the “maleic acid medium” (modified after Doebereiner & Pedrosa, 1992)

without bromine thymol blue, and our “medium B” containing 14 mM KCl, 10 mM HEPES, 7.2 mM NaCl, 5 mM NH_4Cl, 0.5 mM $MgSO_4$, 0.15 mM phosphate (79% K_2HPO_4, 21% NaH_2PO_4 (w/w)), 0.01 mM $CaCl_2$, 0.05% tryptone, 0.0005% yeast and per liter 500 µl trace element solution „SL10“ (Widdel et al., 1983). The pH was 7.0. These agar plates were incubated under the conditions described above for diatom cultivation. Apparently different bacterial colonies were picked with an inoculation loop and streaked at least three times on the same medium to obtain pure cultures. From these cultures, 16S rRNA gene was amplified and sequenced as described below.

The diatom *C. microcephala* was purified from bacterial contaminants by spreading on 1.5% DM agar plates containing 5 $\mu g \cdot ml^{-1}$ tetracycline and 5 $\mu g \cdot ml^{-1}$ kanamycin. The absence of bacterial contaminants was verified by epi-fluorescence microscopy using the dyes 4’,6-diamidino-2-phenylindol (DAPI) or SybrGreen, and by plating on the bacterial media described above.

C. microcephala was co-cultivated with six different bacterial isolates in binary cultures or with all bacterial strains together. As reference, the axenic diatom culture was used. All cultures were grown in 50 ml DM in Erlenmeyer flasks. For every condition and for every harvesting point, three independent culture flasks were prepared. Diatom culture (1.5 ml) with a chlorophyll concentration of 0.31 $\mu g \cdot ml^{-1}$ was used as initial inoculum. Bacteria were added to *C. microcephala* cultures in similar amounts ($OD_{600} \cdot V(\mu l)$ = constant). As a further control, DM in test tubes was inoculated with bacteria as well. Cultures were grown as described above, and chlorophyll content (diatom growth), optical density at 600 nm (OD_{600}), carbohydrate concentration and composition were followed. Samples were taken at four-day intervals over a cultivation period of 32 days.

Biofilms were removed from the glass surface with a rubber spatula, transferred to 50 ml Centrifugation tubes, and suspended. Growth of diatoms and bacteria was measured via

OD_{600}. At least five single measurements were done with every culture, up to 20 if strong biofilms and aggregates were formed. Growth of *C. microcephala* was quantified via chlorophyll contents. From every culture, 1 ml was centrifuged at 16,100 x *g* for 20 min. The pellet was re-suspended in 100 µl methanol and vortexed for 20 min. After addition of 900 µl acetone, particles were spun down again and the chlorophyll content was determined optically (Jeffrey & Humphrey, 1975). For microscopy, 1 ml of every sample was fixed in 10% formaldehyde. For epifluorescence microscopy, samples dried on objective slides were incubated for 5 min with a 1.4 pM DAPI solution, and then washed with water and subsequently with pure ethanol. After evaporation of the ethanol the cells were embedded in AF1 (Citifluor, London).

Treatment of C. microcephala *with spent Medium of the bacterial Strain 32.* Strain 32 was grown in 50% LB medium as described. Cultures were harvested in the stationary phase by centrifugation at 30,000 x *g* for 30 min at room temperature. The supernatant was either filter-sterilized or autoclaved or both, and applied at a ratio of 0.2% - 50% (v/v) to freshly inoculated *C. microcephala* cultures. As a control, 50% LB-medium was added to the diatom cultures. All cultures were grown in three replicates and checked daily by microscopy.

DNA Extraction and 16S rRNA Gene Clone Libraries. Fourteen diatom species (Medlin et al., 2008; Bahulikar & Kroth, 2007) were used in xenic, uni-algal cultures that were sub-cultured at least 4-5 times. For DNA extraction (modified from Murray & Thompson, 1980), cultures were centrifuged, the cell pellet was frozen in liquid nitrogen and crushed with a pestle, mixed with 1 ml of CTAB buffer, and incubated at 65° C for 1 h. The sample was washed with a chloroform-isoamylalcohol mixture 24:1 (v/v). DNA was precipitated with 0.7 volumes isopropanol, washed with 80% ethanol, dried and dissolved in 100 µl 10 mM Tris-HCl, pH 8.0, with 1 mM EDTA. Fifty ng aliquots of DNA were used to amplify 16S rRNA genes using the universal bacterial primers 27f 5’-AGA GTT TGA TCC

TGG CTC AG-3' (Edwards et al., 1989) und 1492r 5'-TAC GGY TAC CTT ACG ACT T-3' (Weisburg et al., 1991). PCR products were purified using the NucleoSpin kit (Macherey-Nagel, Germany) followed by insertion into the pGEM-T vector (Promega, Germany) and transformation into *Escherichia coli* XL1 blue excel (Stratagene, Heidelberg, Germany) according to the manufacturer's protocols. The inserted cloned 16S rRNA genes of 40 - 50 randomly selected colonies per clone library were amplified, digested with MspI (fermentas) according to the manufacturer's instruction, and analyzed for restriction fragment length polymorphism by electrophoresis using 2% Nu-Sieve agarose (NuSieve ® 3:1 Agarose, Cambrex Bio Science Rockland Inc., ME).

Sequencing and phylogenetic Analysis. At least 10% of the cloned 16S rRNA genes showing unique restriction patterns were sequenced. Sequence reactions were prepared either with the dye primer cycle sequencing ready reaction kit (SP6 and T7 primers) or the PRISM ready reaction dideoxy termination cycle sequencing kit (Perkin-Elmer). Sequences were obtained using an Applied Biosystems (model 3700) automated sequencer. Blast searches were performed at NCBI (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990), and closely related sequences were retrieved. All sequences were checked for chimeras by dividing the sequence into two partial sequences and performing blast searches, and phylogenetically analyzed using the ARB software package (version 2.5b; http://www.arb-home.de) (Ludwig et al., 2004). Sequences were added to the ARB database and aligned using the FAST Aligner tool as implemented in ARB. Only sequences larger than 1400 nucleotides were used for alignment. Phylogenetic analysis was done using the maximum likelihood, neighbor-joining and maximum parsimony algorithms.

Chemical Analyses. Nitrate was assayed in cultures after HPLC separation on an A06 column (Sykam) according to the manufacturer's description, and was detected optically at 254 nm wavelength. As standard, 0.01 to 1 mM $Ca(NO_3)_2$ was used.

Polysaccharides were analyzed separately in the soluble and the cell-associated fraction. Cultures were centrifuged at 16°C at 5,250 x *g* for 10 min. The supernatant containing soluble EPS was separated from the pellet. To extract frustule-associated ("bound") EPS, the pellet was re-suspended in 5 ml water and incubated for 1 h in a shaking water bath at 30° C. After centrifugation at 5,250 x *g* for 10 min (Staats et al., 1999) the obtained supernatant contained the bound EPS. Carbohydrate contents of soluble and bound EPS were measured optically using a phenol-sulfuric acid assay (Dubois et al., 1956). As a standard, glucose was used at concentrations from 5 to 500 µg per ml. Polysaccharides were precipitated in 80% (v/v) ethanol at -20° C for at least 12 hours (Staats et al., 1999), centrifuged at 5,252 x *g* and 4°C for 20 min, and dried in a laminar air flow cabinet. Polymers were hydrolyzed at 123°C for 20 min in 2 M trifluoroacetic acid (TFA) (modified from Albersheim et al., 1967). Then the TFA was evaporated, the remaining sugars were dissolved in 1 ml water and analyzed via high-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Jahnel et al., 1998) using equipment from DIONEX. Mixtures of the D-isomers of arabinose, fructose, fucose, galactose, glucose, mannose, ribose and xylose were used as reference compounds.

Results

Analysis, Isolation, and Cultivation of Diatom-associated Bacteria. Single diatom cells were isolated from rocks of the littoral zone of Lake Constance by micromanipulation, and were grown and maintained for two years together with the associated bacteria. 40% of the 16S rRNA genes cloned from diatom cultures were derived from heterotrophic bacteria, and 60% from plastids. Among the bacteria, *Alphaproteobacteria* were dominant (59.2% of all bacterial sequences). *Beta-* and *Gammaproteobacteria* contributed 13% each, the *Bacteroidetes* group 11% and *Verrucomicrobium* spp. 3%. Among the *Alphaproteobacteria*, sequences were related to five different clades (Fig.1). One clade belonged to *Erythromicrobium* and *Porphyrobacter*, two clades belonged to *Sphingomonas*, one to *Brevundimonas* and one to *Azospirillum*. Some sequences were related to *Rhodobacter*.

Betaproteobacteria were mainly related to *Acidovorax* sp. or *Hydrogenophaga sp.* (Fig.1), while most *Gammaproteobacteria* grouped with *Pseudomonas sp.*. One clone grouped with *Aquimonas voraii.*

Within the *Bacteroidetes* group, bacteria were related to *Flavobacterium* or *Sphingobacterium* (Fig.1). Some sequences belonged to *Verrucomicrobia* and *Planctomycetes* (Fig.1).

Figure 1: Phylogenetic tree of 16S rRNA gene sequences obtained from prokaryotic biomass associated with diatom cultures. Clones obtained in our study are denoted as D## followed by the clone number. Representative 16S rRNA gene sequences of cultured and uncultured bacteria were used for the analysis and only sequences of >1400 nucleotides were considered. The tree was calculated by the neighbor-joining method showing 16S rRNA gene sequences recovered from the clone libraries of diatom-associated bacteria. NCBI accession numbers of clones and cultures are given; bar represents 10% divergence. The tree was rooted with *Thermotoga maritima* as the outgroup.

By monthly counting of frustules from biofilms throughout the years 2004 and 2005, *C. microcephala* was found to be one of the dominant benthic diatoms in Lake Constance (data not shown). We cultivated this diatom in uni-algal and in axenic culture. Six strains of heterotrophic bacteria associated with *C. microcephala* were isolated from the non-axenic culture in dilution series. Only strains abundant in 10^5 to 10^7 dilutions were studied further. Strains 28 and 29 were isolated in Doebereiner's medium, strains 30 and 32 in medium B, and strains 31 and 35 in NSY medium. Strains 28, 29, 30 and 31 belonged to the *Alphaproteobacteria*, strain 35 to the *Betaproteobacteria*, and strain 32 to the *Bacteroidetes*.

Co-Cultivation of C. microcephala *with isolated Bacteria.* Co-cultures of *C. microcephala* grown with the isolated associated bacterial strains yielded chlorophyll contents 11% to 66% higher than those of the axenic culture. Within one month, the cells reached chlorophyll concentrations up to 0.9 ± 0.01 µg·ml^{-1}in liquid DM while the axenic cultures yielded a maximal chlorophyll content of 0.5 ± 0.09 µg·ml^{-1} (400,000 cells · ml^{-1} $\pm$ 10%). All co-cultures with bacteria, except those containing strain 32, grew faster than the pure diatom culture. The axenic diatom culture showed maximal growth after twenty days. In the stationary phase, the chlorophyll content remained stable until the end of the cultivation period. In co-cultures with bacteria, the chlorophyll content generally decreased towards the end of the cultivation period (Fig.2).

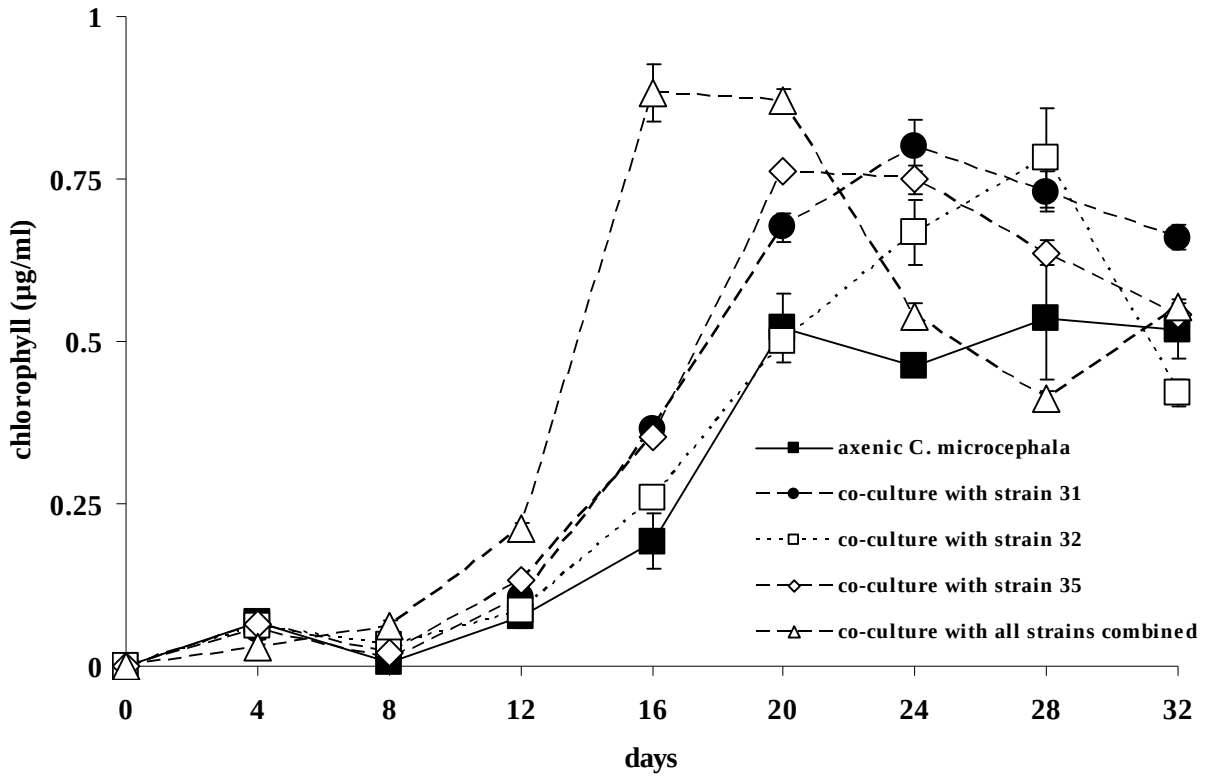

Figure 2: Growth of *Cymbella microcephala* in pure culture (solid line) or in co-culture with bacterial isolates (other lines).

This phenomenon was most distinct in cultures inoculated with all bacterial strains together: the cultures reached maximal cell density and the highest chlorophyll contents of all cultures after only twelve days, followed by a stationary phase lasting for four days before the chlorophyll content decreased (Fig.2/3B). Co-cultures with *Alphaproteobacteria* strains 28, 29, and 30 and with the *Betaproteobacterium* strain 35 reached their maximal chlorophyll concentration at the same time as the axenic culture, the co-culture with the *Alphaproteobacterium* strain 31 four days later, and the co-culture with the *Bacteroidetes*

strain 32 eight days later (Fig.2). In axenic cultures, the OD_{600} values correlated with the chlorophyll content (Fig.3A), and the same was true for the co-cultures with strain 32. The co-cultures with strains 31, 35 and with all bacterial strains together showed an increasing OD_{600} at a time when the chlorophyll content declined (Fig.3B). Similar phenomena were observed with the co-cultures with *Alphaproteobacterium* strains 28, 29 and 30, but to a lesser extent. The initial concentration of free nitrate in DM was 0.34 mM. Until day 28 of cultivation, the axenic diatom used 50% of the nitrate, the co-cultures between 56% and 90%. There was always at least 30 µM nitrate left in all cultures.

Quantification of Carbohydrate Formation. All *Proteobacteria* enhanced polysaccharide secretion by the diatom. The axenic culture reached concentrations up to 121 $\mu g \cdot ml^{-1}$ soluble carbohydrates (up to 284 pg per diatom cell) whereas in all co-cultures with *Proteobacteria*, the respective amount increased up to 226 $\mu g \cdot ml^{-1}$ or up to 444 pg per diatom cell (co-culture with strain 35) (Tab.1). The co-culture with the *Bacteroidetes* strain showed a decreased polysaccharide concentration during the cultivation period, and lower polysaccharide contents were observed also in the co-cultures with all bacterial isolates combined (see Tab.1).

Bound carbohydrates were formed by diatoms in axenic culture to a maximal concentration of 2.5 $\mu g \cdot ml^{-1}$ (~ 6 pg per diatom cell) whereas co-cultures with the *Betaproteobacterium* strain 35 reached 4.1 $\mu g \cdot ml^{-1}$ (~ 7 pg per diatom cell). The maximal concentration of bound carbohydrates varied from 2.1 $\mu g \cdot ml^{-1}$ to 2.7 $\mu g \cdot ml^{-1}$ for co-cultures with *Alphaproteobacteria*. All co-cultures with *Alphaproteobacteria* showed a decreasing ratio of bound carbohydrates to diatom cell number towards the end of the cultivation period. Maximum formation of bound carbohydrates was observed in cultures with the *Bacteroidetes* strain 32 and in that with all bacteria together (Tab.1). In both cultures, the ratio of bound

carbohydrates to diatom cell number increased strongly towards the end of the cultivation period.

Table 1: Carbohydrate contents of growing *C. microcephala* cultures on day 20 and 28 after inoculation, given as µg carbohydrates per milliliter culture and pg carbohydrates per diatom cell.

	Amount of soluble carbohydrates				**Amount of bound carbohydrates**			
	day 20		**day 28**		**day 20**		**day 28**	
added bacterial strain	**µg·ml^{-1}**	**pg · cell^{-1}**	**µg·ml^{-1}**	**pg · cell^{-1}**	**µg·ml^{-1}**	**pg · cell^{-1}**	**µg·ml^{-1}**	**pg · cell^{-1}**
-	46.3 ± 2.8	111	121 ± 18.2	284	1.6 ± 0.2	3.8	2.5 ± 0.3	5.9
***Alphaproteobacterium* strain 28**	113 ± 4.5	209	171 ± 1.6	408	2.7 ± 0.6	5	1.6 ± 0.1	3.9
***Alphaproteobacterium* strain 29**	93.1 ± 7.3	199	162 ± 13	407	2.2 ± 0.2	4.8	1.2	3.1
***Alphaproteobacterium* strain 30**	79.2 ± 10.1	170	167 ± 9.5	310	2.1 ± 0.2	4.5	1.8 ± 0.1	3.3
***Alphaproteobacterium* strain 31**	74.9 ± 2.7	139	192 ± 12	328	2.1 ± 0.1	3.8	2.1 ± 0.2	3.6
***Bacteroidetes* strain 32**	33.2 ± 0.4	83	74.5 ± 6.7	119	4.4 ± 0.5	12.2	7 ± 0.6	11.2
***Betaproteobacterium* strain 35**	78.2 ± 3	129	226 ± 13.7	444	4.1 ± 0.6	6.8	2.7	5.2
All	38.4 ± 2.5	55.1	103 ± 18	312	2.8 ± 1	4	14	42.5

Chlorophyll and soluble carbohydrate concentrations in axenic *C. microcephala* cultures correlated, with the polysaccharide content slightly retarded to the chlorophyll content (Fig.3C). This was true for all cultures, except the co-cultures with strain 32, where both graphs nearly coincided (Fig.3D). In the co-cultures with the *Betaproteobacterium* strain 35 and those with all bacterial strains, a stagnating optical density was followed by a decline of the concentration of soluble carbohydrates by 81% within the last four days (Fig.3E).

The concentrations of bound carbohydrates within axenic *C. microcephala* cultures correlated with the chlorophyll concentrations and with the OD_{600} (Fig.3A/F). This was also true for co-cultures with strains 32 and 35. With all co-cultures incubated with

Alphaproteobacterium strains (strains 28, 29, 30 and 31), a parallel increase of the chlorophyll concentrations and the content of bound carbohydrates was observed, but at the same time the level of bound EPS decreased when the OD_{600} stagnated or increased (Fig.3G/H).

The situation was entirely different with the co-cultures inoculated with all bacteria together. Here the bound carbohydrate contents correlated with the OD_{600}, but not with the chlorophyll content. While the latter decreased, more cell-associated carbohydrates were found (data not shown).

The amounts of bound and soluble carbohydrates were strictly correlated. An increase of bound carbohydrates was followed approximately eight days later by an increase in soluble carbohydrates (Fig.3I). This was true for the axenic culture and for nearly all co-cultures, with the exception of the co-cultures with strain 32 and that with all bacterial strains together. Here, both carbohydrate fractions increased and decreased simultaneously (Fig.3J).

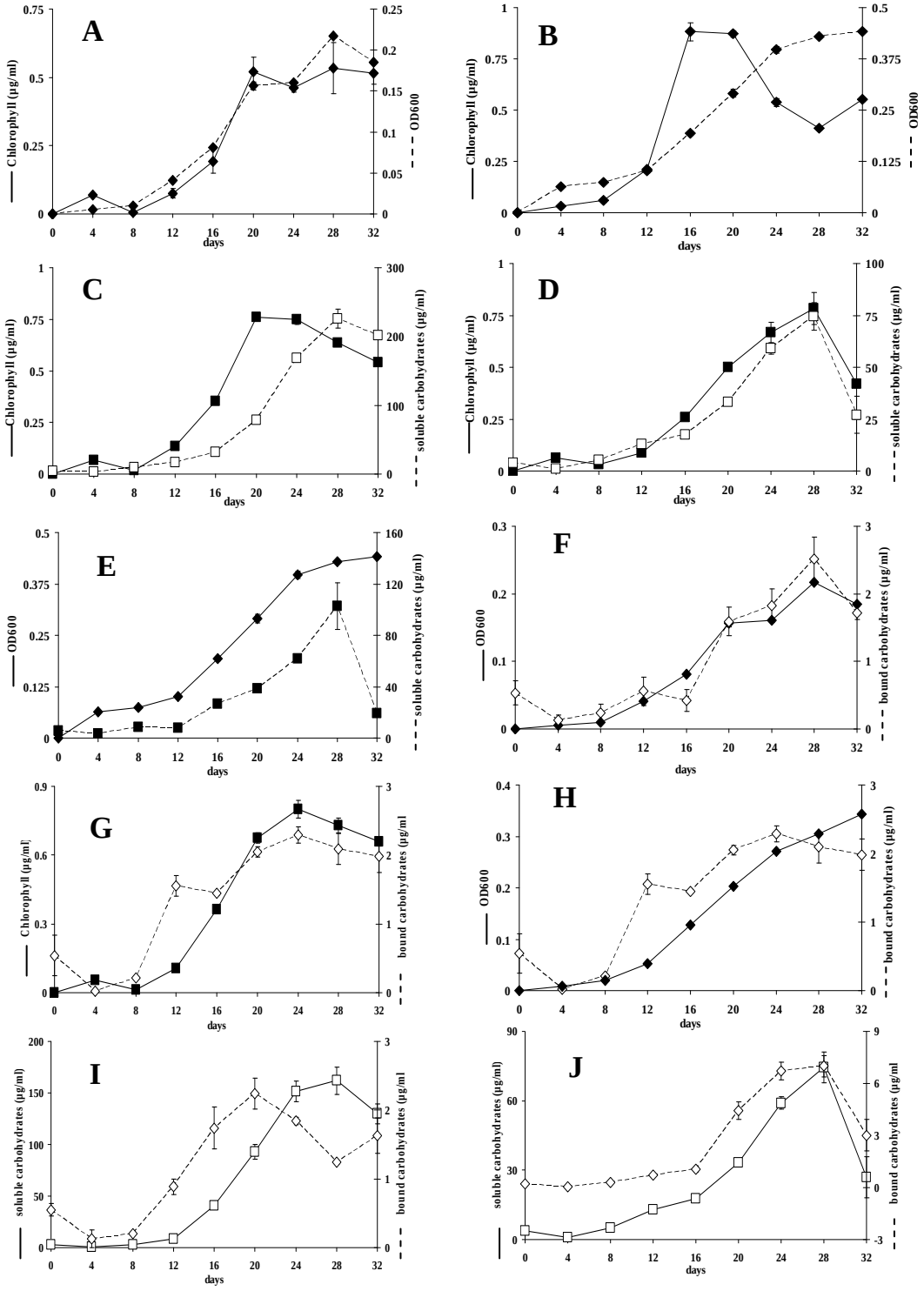

Figure 3: Growth and product formation by *C. microcephala* in pure culture and in co-cultures. **A/B:** Growth of axenic *Cymbella microcephala* cultures (A) and co-cultures with mixed bacterial strains (B) measured as OD_{600} (dashed line) and chlorophyll content (solid line) **C/D**: Chlorophyll content (solid line) and concentrations of soluble carbohydrates (dashed line) of *C. microcephala* co-cultures with strain 35 (C) or 32 (D) **E**: OD_{600} (solid line) and concentration of soluble carbohydrates (dashed line) of *C. microcephala* co-cultivated with all bacterial isolates **F**: OD_{600} (solid line) and concentrations of bound carbohydrates (dashed lines) of axenic *C. microcephala* **G/H:** Co-culture of *C. microcephala* and strain 31: chlorophyll content (solid line, G), OD_{600} (solid line, H) and concentrations of bound carbohydrates (dashed line) **I/J:** Concentrations of bound (dashed line) and soluble (solid line) carbohydrates from co-cultures with *C. microcephala* and strain 29 (I) or strain 32 (J)

Analysis of Carbohydrate Composition. Soluble carbohydrates isolated from axenic *C. microcephala* cultures contained 40 to 50% galactose and 30 to 40% mannose/xylose monomers. Furthermore, about 7.2% rhamnose and 3.5% fucose were detected throughout the cultivation period. The level of mannose/xylose decreased slightly whereas the galactose content increased towards the middle of the cultivation period. After 16 days, the proportion of galactose decreased and that of mannose/xylose increased. The glucose content was constantly reduced until complete absence after 20 days of cultivation. Similar results were observed for N-acetyl-D-glucosamine (GlcNac) which decreased until day 16 of cultivation. Arabinose and fructose proportions were below one percent and fluctuated randomly.

All co-cultures with the different bacteria showed similar proportions of monomers, whereas the described fluctuations of mannose/xylose and of galactose were always larger, as with the axenic culture. The strongest decrease of mannose/xylose was found in the co-cultures with all bacterial strains together, reaching a minimum of 13.2% on day 16 of cultivation; the galactose proportion increased simultaneously to a maximum of 74.1%. The proportions of glucose, GlcNac, rhamnose and fucose were generally similar to that in the axenic culture in all situations. All other monomers showed fluctuated randomly within a proportion of 1.5%.

Cell-associated polysaccharides of the axenic *C. microcephala* cultures contained a similar percentage of mannose/xylose (30-40%) and of galactose (50-60%) as did the soluble carbohydrates. Different from the soluble carbohydrates, relevant proportions of glucose were found also towards the end of the cultivation period in the bound polysaccharides of the axenic cultures (4.7% at day 32). Arabinose and fructose were absent; fucose, rhamnose and GlcNac fluctuated at low proportions. In co-cultures with strain 32 or those with all bacterial strains together, GlcNac was missing. Co-cultures either showed a decrease in glucose content (strains 29 and 30), complete absence of this monomer (co-cultures with all strains), or the

proportion fluctuated randomly. In all co-cultures, the proportions of fucose (up to 7%) did not change throughout the cultivation period, different from the axenic culture. All other monomers showed fluctuations at a lower level; fructose was never detected.

Co-Cultures of C. microcephala *with the* Bacteroidetes *Strain 32. C. microcephala* co-cultures with *Bacteroidetes* strain 32 formed a polysaccharide capsule surrounding the diatom cells with an estimated volume of up to ten times that of the algal cell. This capsule was stained by DAPI (Fig.4F) and was also visible in phase-contrast microscopy. Axenic *C. microcephala* cells did not form these capsules. The concentration of soluble carbohydrates was the lowest one of all co-cultures containing single bacteria whereas the concentration of bound carbohydrates was highest. The soluble fraction showed a higher level of fucose and rhamnose, the bound fraction had the lowest fucose content, and GlcNac was completely absent.

Capsule formation was induced also after addition of bacterial culture supernatant. After 3 - 4 days of incubation, the first algal cells were surrounded by this gel-like matrix. Also filter-sterilized or autoclaved culture supernatant added at 0.2% to 10% (v/v) ratio caused this effect. No difference was observed between frozen and fresh bacterial supernatant. This effect was caused neither by the medium (50% LB) itself nor by the pH of the culture supernatant (6.9-7.3).

Co-Cultures and Biofilm Structure. Epifluorescence microscopy of DAPI preparations revealed that, depending on the bacterium in every defined co-culture combination, different cell aggregates and biofilm structures developed (Fig.4). Exclusively *Betaproteobacterium* strain 35 grew suspended, showing visible turbidity during co-cultivation. The characteristic visual patterns regarding cell aggregation, such as turbidity, biofilm or capsule formation in co-cultures of *C. microcephala* with single bacterial strains, were observed also in the mixed cultures.

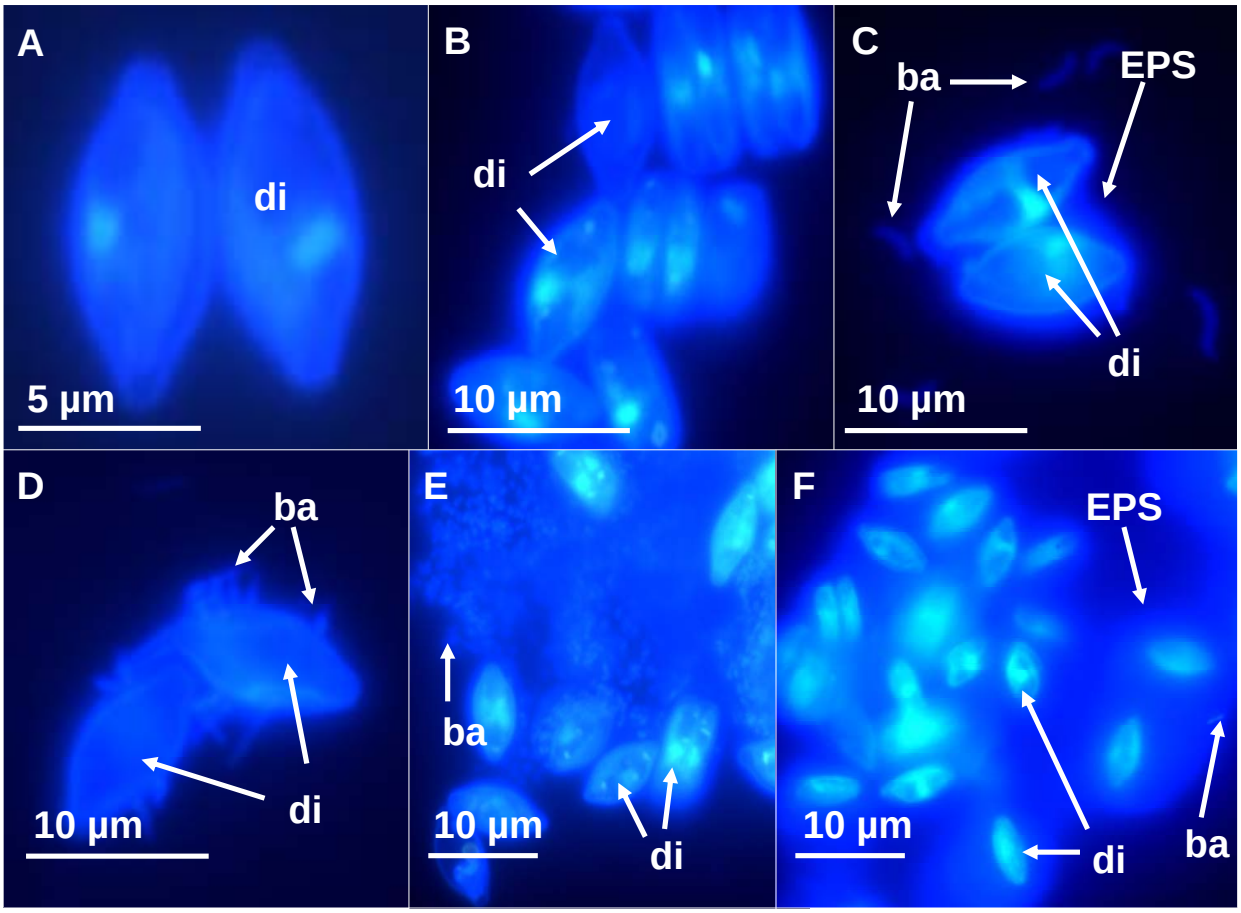

Fig. 4: Cell-cell aggregates formed by *C. microcephala* (di) under different culture conditions. Epifluorescence photomicrographs of DAPI preparations of *C. microcephala* cells grown either axenic (A), or co-cultivated with strain 28 (B), 29 (C), 30 (D), 31 (E) or 32 (F). In all Co-cultures, bacteria (ba) show a strain-specific assembly in relation to the diatom. In co-cultures with strain 32 (F), *C. microcephala* secretes an EPS capsule.

Discussion

Phylogenetic Analysis of Diatom-associated Bacteria. Although the diatoms used in this study represented different genera the associated bacteria often exhibited striking similarities of their 16S rRNA gene sequences. They were dominated by *Alphaproteobacteria* as also reported earlier (Grossart et al., 2005; Riemann et al., 2000). Bacterial communities associated with aggregates from planktonic diatom blooms in Lake Constance were dominated by *Alpha-* and *Betaproteobacteria* and by *Bacteroidetes* as well (Schweitzer et al., 2001). The dominant 16S rRNA gene sequences grouped with *Sphingomonas*, *Caulobacter*

and *Rhizomonas* or with *Brevundimonas* and *Mycoplasma,* and formed a clade with databank sequences obtained from lake snow microaggregates (Simon et al., 2002). Others were similar to sequences of *Roseobacter,* including *Azospirillum*-related sequences that were described earlier to be associated with marine diatom assemblages (Allgaier et al., 2003). Sequences of *Betaproteobacteria* in this study were related to *Hydrogenophaga* and *Acidovorax* that had also been found in diatom-derived micro-aggregates (Brachvogel et al., 2001) and in lake snow of Lake Constance (Schweitzer et al., 2001). Most of the sequences of *Gammaproteobacteria* were related to the eel pathogen *Pseudomonas anguilliseptica* (Doménech et al., 1999). 16S rRNA gene sequences related to *Bacteroidetes* were often amplified from diatom cultures. These sequences were found in epilithic biofilms in Lake Constance (data not shown) and appear to be associated frequently with diatoms (Grossart et al., 2005; Knoll et al., 2001; Riemann et al., 2000). Interestingly, the abundant types of 16S rRNA gene sequences derived from our samples have recent common ancestors, although the tested diatoms were phylogenetically highly diverse including raphid and araphid species. Since other 16S rRNA gene sequences derived from diatom-associated prokaryotes (Schäfer et al., 2002; Riemann et al., 2000; Bowman et al., 1997) confirm this observation, diatoms might be regarded generally as a micro-habitat to which especially *Proteobacteria* and *Bacteroidetes* have adapted and evolved separately, independent whether the diatoms were planktonic or benthic, raphid or araphid, freshwater- or saltwater-adapted, or terrestrial or found in polar ice.

Co-Cultivation of C. microcephala *with bacterial Isolates.* In our co-cultures with the ubiquitous freshwater diatom *C. microcephala,* we showed that the diatoms produced the organic carbon source for these bacteria. Further we confirm studies in which heterotrophic bacteria supported diatom growth (Grossart, 1999; Fukami et al., 1997) although opposite observations were reported as well (Baker & Herson, 1978). Apparently the bacteria release

substances that support growth of *C. microcephala*, or they consume substances that might otherwise inhibit diatom growth. Since nitrate availability was not a limiting factor, bacterial N_2 fixation can be ruled out as a possible means of support.

The measured OD_{600} values can be regarded as a rough estimate of the total cell numbers including diatoms and bacteria. Graphs of chlorophyll concentrations and OD_{600} coincide with axenic cultures, thus confirming the reliability of both methods. An increasing OD_{600} and simultaneous decreasing chlorophyll contents should be due to increased bacterial growth. Increasing bacterial growth while diatom growth stagnates can be explained either as bacterial exploitation of a substrate derived from the diatom, e.g., glycolate, or secreted polymeric organic matter (Grossart & Simon, 2007; Grossart et al., 2006), or phosphate released from dead diatoms. In co-cultures with the *Bacteroidetes* strain 32, the OD_{600} and the chlorophyll content increased and decreased in parallel. Obviously, bacteria and diatoms grew simultaneously, either due to the production of an unknown growth-supporting factor, or by a direct bacterial influence on the growth of *C. microcephala*.

Formation of Carbohydrates. The fraction of soluble carbohydrates contained glucose that derived from soil extract that was added to the culture medium. During co-cultivation glucose disappeared completely from the soluble fraction, probably due to bacterial consumption. However, glucose disappeared also in the axenic culture and was not detectable after 16 days. Obviously, it was consumed or converted also by the diatom. A similar phenomenon was observed with GlcNac.

All *Proteobacteria* in this study enhanced secretion of soluble polysaccharides by the diatom, probably caused by an unknown bacterial factor influencing the diatom. In the axenic cultures and in most co-cultures diatom growth was followed by the accumulation of secreted carbohydrates in the medium. This was not the case in co-cultures with strain 35 or in those with all strains together. Here, a decrease of soluble carbohydrates, an increasing OD_{600}, and

rising concentrations of frustule-associated carbohydrates indicate that soluble carbohydrates were preferentially used by the *Betaproteobacterium* strain 35. Interestingly, the co-culture with strain 35 produced the highest amounts of soluble carbohydrates. *Betaproteobacteria* are typically found attached to lake snow often rich in dead or dying diatoms (Brachvogel et al., 2001; Schweitzer et al., 2001) in the water column of Lake Constance. Therefore, these bacteria are likely to utilize dissolved polymers and to degrade dead algal cells. Strain 35 grew freely suspended in the culture flask, indicating that it might have been found only accidentally in the biofilm.

The *Alphaproteobacteria* appear to utilize cell-bound polysaccharides as their carbon source. In these co-cultures, the content of cell-associated carbohydrates decreased simultaneously with the chlorophyll content, while at the same time the OD_{600} increased and soluble sugars started to accumulate. *Alphaproteobacteria* are known to be associated ubiquitously with diatoms, independent of the habitat of the algae (Grossart et al., 2005; Makk et al., 2003; Schäfer et al., 2002; Brachvogel et al., 2001; Knoll et al., 2001; Riemann et al., 2000; Bowman et al., 1997; Weiss et al., 1996). Adaptation of *Alphaproteobacteria* to this habitat appears likely. They might feed on frustule-associated organic matter because these carbohydrates are permanently produced by the alga and do not diffuse into the surrounding water column. This hypothesis is supported by the observation that these *Alphaproteobacteria* were found to be mainly embedded in the diatom/bacteria biofilms.

The Bacteroidetes *Strain 32 Influences the Secretion of Diatom Carbohydrates via soluble Molecules.* The *Bacteroidetes* strain 32 apparently strongly influenced the diatom carbohydrates by decreasing the content of soluble polysaccharides drastically and increasing the level of bound polysaccharides. Microscopic observation showed the formation of capsules around the diatoms. Low concentrations of soluble EPS and high amounts of bound EPS were observed also in co-cultures with all bacteria. *Bacteroidetes* are often found on

diatom-rich detritus (Knoll et al., 2001), e. g., in diatom cultures in the late stationary phase (Grossart et al., 2005; Riemann et al., 2000). They degrade complex polymers (Kirchman, 2002; Shewan & Mc Meekin, 1983) and colonize solid substrates rapidly (Pinhassi et al., 1999). In microscopic observations, we often found these bacteria on the surfaces of capsules surrounding the diatoms. The observed decrease of soluble sugars could be caused by immediate bacterial consumption or by reduced secretion of soluble EPS to form preferentially bound carbohydrates. Since this effect can also be induced by autoclaved bacterial culture supernatant we assume that the bacterium produces a thermostable molecule which induces capsule secretion, possibly as a protection against any kind of threat (e.g. predation, toxins).

Single bacterial Strains and mixed Bacteria in Co-Culture with C. microcephala. Microscopic observation of the co-cultures showed the formation of specific structures of cell aggregates of *C. microcephala* and single bacterial strains (Fig.4). In the co-cultures with mixed bacteria, all types of such structures were found. Specific effects of isolate 32 on diatom carbohydrates, namely, high amounts of frustule-associated carbohydrates (capsule formation) and low concentrations of soluble carbohydrates, were also measured in the mixed co-cultures. Every single strain appears to use a substrate deriving directly from the diatoms, thereby forming characteristic aggregates together with the diatom, no matter whether other bacteria are present or not. Obviously, the diatom provides various niches for different bacteria, and benefits from their presence. In natural biofilms, such niche formation might, beside other factors, explain the success and distribution pattern of certain diatoms and associated bacteria. The microorganisms may adapt to each other and create a kind of microenvironment optimized for interacting partners. Diatoms and bacteria might support each other by an equilibrium of cross-feeding, possibly optimized by exchange of chemical factors. Such associations can be specific or random. It is likely that cross-feeding partners

may change due to various factors such as cell density, presence of other microorganisms and their secretions, availability of nutrients, or abiotic factors such as light, temperature, water currents etc. Further such interactions appear to initialize formation of diatom biofilms and aggregates, as this was shown so far with marine microbial communities (Grossart & Simon, 2007; Grossart et al., 2006).

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Bacterial Influences on Growth and Carbohydrate Secretion of representative epilithic Diatoms and Correlations with dissolved free Amino Acids

Chapter 3

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Key words: diatoms, bacteria, interaction, biofilm, amino acid, EPS

Abstract

Diatom carbohydrate secretion is an important factor in phototrophic, epilithic biofilms. Such biofilms also harbor a distinct assemblage of heterotrophic bacteria. In this study bacterial effects on carbohydrate secretion of nine representative diatom species were investigated. The presence of bacteria affected cell density in cultures of most diatoms. Moreover, bacteria had an influence on growth rates and carbohydrate secretions of all diatoms. Our studies revealed strain-specific effects of bacteria on exo-carbohydrate production by epilithic diatoms, indicating that the quantity of extracellular carbohydrates in biofilms dominated by epilithic diatoms is related to the respective bacterial community composition. Concentrations of a variety of dissolved free amino acids detected in diatom/bacteria co-cultures correlated with observed bacterial effects on diatom growth. Thereby raphid diatoms generally benefited more from the presence of numerous bacteria than araphid diatoms. We hypothesize, that amino acids exuded by the diatoms can cause auto-inhibition of diatom growth when not degraded by bacteria. On the other hand, bacteria may have a direct influence on amino acid secretion by the diatoms, or bacterial production of specific amino acids potentially regulates diatom growth as well, either via degradation of proteins, or by direct secretion.

Introduction

Epilithic biofilms are complex communities, based on interactions between primary producers (e.g., algae and cyanobacteria) and heterotrophic organisms, such as bacteria, fungi, protozoa, insects, larvae, shellfish etc. (Makk et al., 2003). Freshwater biofilms in the littoral zone of Lake Constance, a large mesotrophic, warm-monomictic lake in central Europe, are dominated by photoautotrophic primary producers like epilithic diatoms (Bahulikar, R., unpublished data). In addition, heterotrophic bacteria are found to be embedded in the biofilm matrix. Extracellular polymeric substances (EPS) are a major component of the biofilm matrix. Polymer chemistry and the surface properties of EPS are thought to affect coagulation and aggregation (Bhaskar et al., 2005), water congestion (Potts, 1994) and ion binding (Chin et al., 1991). Furthermore, EPS can act as a kind of glue stabilizing whole sediments and preventing watersides from erosion (Stal & Brouwer, 2003; Wigglesworth-Cooksey et al, 2001; Decho, 2000; Sutherland & Grant, 1998). Many diatoms produce large amounts of sticky mucilage, consisting mainly of polysaccharides, which stabilize the biofilm matrix. Parts of the EPS are soluble, parts are associated with living cells or the substratum, forming jelly-like to solid structures (bound EPS). These EPS are produced for various assignments, e.g. raphid diatoms secrete polysaccharides and glycoproteins for movement (Graham & Wilcox, 2000; Pickett-Heaps, 1991), for attachment to the substrate or the formation of cell aggregates, capsules, stalks, pseudo filamentous tubes, fibrils etc. (Hoagland et al., 1993). Recently, it has been shown that also extracellular proteins, often highly glycosilated, are involved in mucus production and diatom adhesion (Dugdale et al., 2006; Chiovitti et al., 2003). EPS secretion is not only favorable for an individual diatom cell but also an important factor for multi-cellular biofilm formation, as shown by Bruckner et al. (2008). EPS can be also used by heterotrophic organisms as a carbon source (Haynes et al., 2007; Giroldo et al., 2003). It has been shown that bacteria can affect adhesion strength of diatoms in multiple

ways: reduction of adhesion, (Wigglesworth-Cooksey & Cooksey, 2005), enhancement (Grossart, 1999) or even both effects depending on the cultivation parameters (Gawne et al., 1998). These changes are accompanied by a qualitative change in biofilm EPS composition (Grossart, 1999; Wigglesworth-Cooksey & Cooksey, 2005).

Earlier, we have shown that the monomer composition of EPS of a ubiquitous epilithic freshwater diatom, *Cymbella microcephala* Grunow, is affected by bacteria but often only to a small extent (Bruckner et al., 2008). However, bacteria had a pronounced effect on the quantity of EPS produced by *C. microcephala*. Grossart et al. (2006) also report on strong influences by living bacteria on the amount of community EPS in diatom/bacteria co-cultures. To proof whether bacterial influences on diatom carbohydrate secretion are of ecological relevance, we have monitored carbohydrate dynamics of five raphid and four araphid biofilm forming benthic freshwater diatom strains.

Other earlier observations suggest that growth of *C. microcephala* is strongly enhanced by numerous bacteria. In the literature, however, bacterial influences on diatom growth remain ambiguous since diatom growth may be suppressed or enhanced by single bacteria or bacterial communities (Fukami et al., 1997; Hirayama & Hirayama, 1997; Baker & Herson, 1978). Here we have studied effects of bacteria on growth of nine benthic freshwater diatom strains and two strains of a model diatom, *Phaeodactylum tricornutum* Bohlin. Seven of our strains belonged to raphid and four strains to araphid diatoms. We wanted to test, whether growth patterns of different diatom species change in the presence of bacteria and whether a specific bacterial strain causes the same or even different effects to our diatom cultures. In addition we wanted to know, whether such effects derive from particular soluble substances of bacterial origin. We tested this effect by adding bacterial spent medium to the diatom cultures.

Since biofilms are very complex communities, they often exhibit a high phenotypic plurality regarding growth, substrate and nutrient utilization, EPS production and

cell/cell communication, resulting in surprisingly coordinated multicellular behavior, even perceived as "city of microbes" (Watnick & Kolter, 2000). Integrating opportunistic individuals in coordinated units has been assumed to be mediated by quorum sensing (Dunlap, 1997; Fuqua et al., 1996; Ruby, 1996). In addition to quorum sensing other signaling or regulatory events can be triggered in such communities by extracellular soluble substances either of algal or bacterial origin, e.g. dissolved free amino acids (DFAA). Although DFAA can be an important extracellular factor for interactions between diatoms and bacteria (Grossart et al., 2006), not much is known about the effect of DFAA on diatom growth. *Chaetocerus debile*, *C. affinis* and *Thalassiosira rotula* were described to release amino acids during different growth phases (Myklestad et al., 1989; Hammer & Brockmann, 1983; Poulet & Martin-Jézéquel, 1983). The utilization of DFAA by diatoms as a nitrogen source has been discussed controversially (Flynn and Butler, 1986). Thus, in this study we have investigated the effects of DFAA on diatom growth detected in defined co-cultures of diatoms and bacteria and in axenic diatom cultures treated with artificial DFAA pools. We postulate that degradation and secretion of DFAA by bacteria influence diatom growth in a species-specific manner.

Materials and Methods

Cultures: All our experiments were performed with the epilithic freshwater biofilm diatoms *Achnanthes minutissima* Kützing, *Cymbella minuta* Hilse ex Rabenhorst (two strains), *Cymbella microcephala*, *Fragilaria pinnata* Ehrenberg, *Pseudostaurosira* sp. D. M. Williams & F. E. Round, *Punctastriata* sp. D. M. Williams & F. E. Round, *Staurosira* sp. C. G. Ehrenberg (Medlin et al., 2008; Bahulikar & Kroth, 2007), and the unidentified diatom strain D164. Furthermore, the model diatoms *Phaeodactylum tricornutum* Utex 646 and

CCAP1055/1 were used. Standard conditions for cultures were 50 µE light intensity for 16 h daily at 16°C. As culture vessels either Erlenmeyer flasks (100 ml) or 24-well plates were used. For co-cultivation with the diatoms we used the *Alphaproteobacteria* strains 29 and 31 and *Bacteroidetes* strain 32, isolated from cultures of a xenic epilithic freshwater diatom (for detailed description see Bruckner et al., 2008). Additionally the model bacterium *Escherichia coli* (strains K12 MG1655 and XL1 blue) was used. Diatom cultures and co-cultures with bacteria were grown in diatom medium (DM) (Watanabe, 2005), containing 1.6% (w/v) NaCl for *P. tricornutum* strains. Additionally diatoms were grown in pure culture and treated with 2% (v/v) culture supernatants from the above mentioned bacteria. All bacteria were grown in LB or half strength LB. The spent bacterial medium was sterile-filtered through syringe tip filters with 0.2 µm pore size. Selected diatoms were also grown with 0.1 $g \cdot l^{-1}$ autoclaved casein-peptone which served as an artificial DFAA pool.

Growth Curves: All samples were taken in three replicates. Biofilms were removed from surfaces and were suspended by careful shaking. Growth of the diatoms was quantified via chlorophyll a concentration (*chl a*). Correlations between *chl a* and cell density were proofed by microscopic counting at random sampling times. Samples were centrifuged at 16,100 *g* for 10 min. The resulting pellets were suspended in methanol, vortexed for 20 min and 9 volumes acetone were added. Particles were spun down again and *chl a* was determined optically (Jeffrey & Humphrey, 1975).

Carbohydrates: For analyzing the content of soluble carbohydrates, cultures were centrifuged at 16°C at 5,250 *g* for 10 min. The supernatants containing the soluble EPS were carefully separated from the pellets. Carbohydrate contents were measured optically using a phenol-sulfuric acid assay (Dubois et al., 1956). As a standard, glucose was used at concentrations from 5 to 500 $\mu g \cdot ml^{-1}$. The carbohydrate monomer composition of random samples from every culture situation was analyzed by HPAE-PAD (Bruckner et al., 2008, Jahnel et al., 1998)

Amino Acids: For analyzing the content of dissolved free amino acids (DFAA), cells were centrifuged at 16°C at 5,250 *g* for 10 min. The supernatant containing DFAA and dissolved combined amino acids (DCAA) was separated from the pellet and frozen at -80°C until analysis. For further analyses replicates were combined. In addition to our cultures, we quantified the amino acid content in pore water from natural diatom dominated epilithic biofilms of Lake Constance. We distinguished between thin, young biofilms (< 1 mm, ~ 6 months old) and thick, mature biofilms (> 5mm, ~ 23 months old). Biofilms were scrapped from rocks and treated as described for the cultures. All samples were filtered through 0.22 µm pore size low protein binding acrodisc filters (Pall Corporation). Concentrations of DFAA were analyzed by HPLC after ortho-phthaldialdehyde derivatization (Lindroth and Mopper, 1979, modified by Grossart et al., 2007). Dissolved combined amino acids (DCAA) were hydrolyzed with 6 N HCl at 160°C for 1 h and analyzed as DFAA.

Results

Growth Curves and DFAA Concentrations: Cell Density

Achnanthes minutissima: Axenic cultures reached a maximal *chl a* concentration of 1.91 µg·ml^{-1} after 24 days of incubation (Fig.1). Co-cultures with the *Bacteroidetes* strain 32, yielded much lower maximal *chl a* contents (0.89 µg·ml^{-1}) (Fig.1). Co-cultures with *Proteobacteria* reached a lower maximal *chl a* concentration (1.67 and 1.68 µg·ml^{-1}) (Fig.1). Following the concentration of DFAA in these cultures in the spent culture medium, interestingly, the reduced cell density of *A. minutissima* in co-culture with bacteria occurred in parallel to lower concentrations of 6 different amino acids in the stationary growth phase of xenic than in that of axenic cultures (Tab.1).

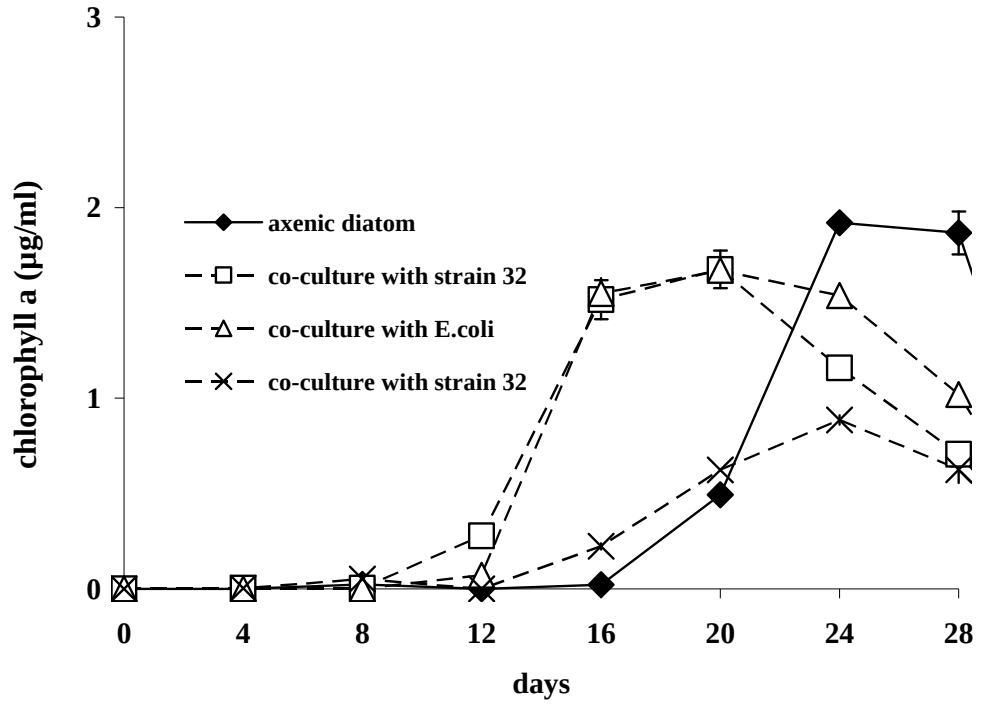

Figure 1: Growth of axenic *A. minutissima* and in co-culture with different bacteria. In co-culture all bacteria reduce diatom cell density compared to the axenic strain, but enhance diatom growth rate.

Cymbella minuta: Axenic cultures of strain I147 reached maximal *chl a* concentrations of 2.57 µg·ml^{-1} after 20 days. Co-cultures with bacteria showed lower maximal chlorophyll contents (up to 2.31 µg·ml^{-1}) (similar to *A. minutissima*, Fig.1). Cultures treated with 2% spent medium from strain 32 or XL1 blue did not grow at all. Reduced growth of *C. minuta* I147 in co-culture with bacteria occurred in parallel to lower DFAA concentrations in the spent culture medium during the stationary growth phase of xenic than in that of axenic cultures. Similar interrelations were found for 9 amino acid monomers (Tab.1).

The axenic cultures of *C. minuta* strain B7 reached a maximal *chl a* content of 1.98 µg·ml^{-1} after 24 days of incubation (Fig.2). Co-cultures with bacteria reached higher maximal *chl a* concentrations (up to 2.39 µg·ml^{-1}) (Fig.2). Measurable diatom growth in co-culture with bacteria started earlier than in axenic cultures (Fig.2). The increased cell density

of *C. minuta* B7 in co-culture with bacteria occurred in parallel to decreased DFAA concentrations in the growth medium at the stationary growth phase of xenic than in that of axenic cultures. Similar relationships were found for 9 amino acid monomers (Tab.1).

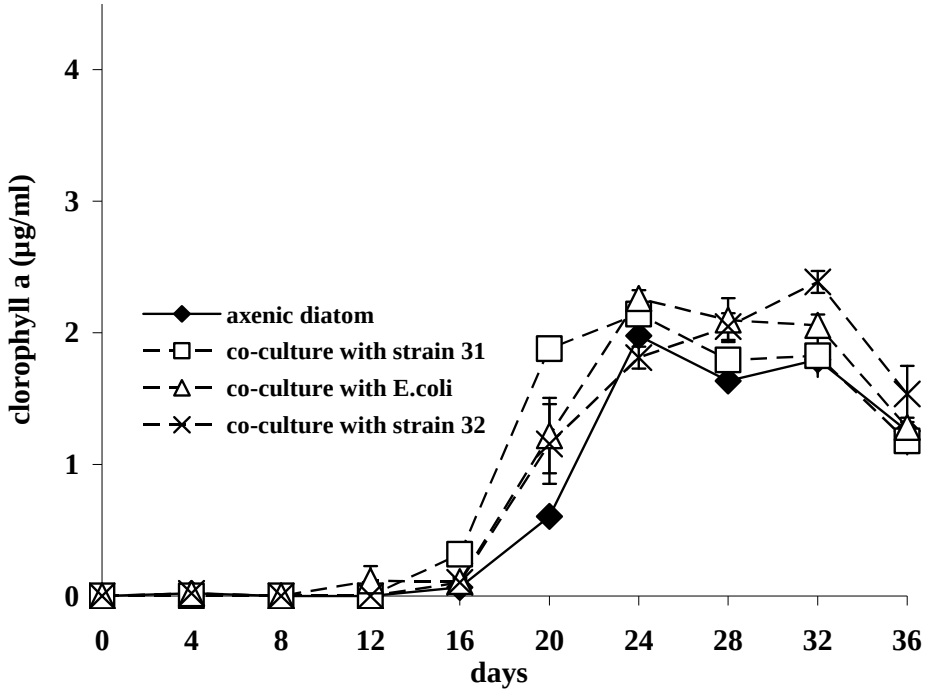

Figure 2: Growth of axenic *C. minuta* B7 and in co-culture with different bacteria. In co-culture all bacteria increase diatom cell density compared to the axenic strain and enhance diatom growth rate.

Table 1: Concentrations of DFAA (nM) in the stationary growth phase detected in spent culture media of axenic diatom cultures and diatom bacteria co-cultures. Likelihood (F), that differences in amino acid composition between the presented DFAA pools from co-cultures compared to the axenic diatoms are not significant, was proofed by F-test.

	axenic	**co-culture with strain 31**	**co-culture with *E. coli***	**co-culture with strain 32**
DFAA conc. (nM)	***Achnanthes minutissima***	F = 0.000	F = 0.000	F = 0.023
His	1935.3	182.7	163.1	737.7
Arg/Cit	6.2	0.1	0.2	3.4
Tyr	1848.5	13.2	23.0	145.1
Met	71.9	23.0	14.1	21.9
Phe	226.0	26.4	13.4	44.9
Leu	1188.8	37.0	9.1	179.4
	Cymbella minuta I147	F = 0.000	F = 0.003	F = 0.064
all DFAA	25008.8	5691.9	8306.8	12607.3
Glu	6912.9	27.8	209.5	3968.6
His	1707.6	798.0	889.8	796.1
Ser	550.1	152.0	409.7	195.3
Arg/Cit	32.3	0.1	1.5	19.6
Gly/Thr	5624.6	448.8	812.3	1485.2
Ala	196.9	77.1	82.1	38.9
Tyr	1946.4	119.9	534.7	585.0
Met	2873.2	162.0	80.4	56.7
Val	672.3	133.3	55.6	51.1
	Cymbella minuta B7	F = 0.004	F = 0.002	F = 0.848
all DFAA	3723.8	1273.9	1115.1	3450.7
Asp	496.6	70.6	28.4	471.5
His	524.8	374.9	315.8	377.1
Ser	105.3	54.8	63.8	59.1
Arg/Cit	5.6	0.3	0.4	4.2
Gly/Thr	102.9	75.0	55.9	96.0
Tyr	597.2	114.3	60.3	480.8
Met	114.6	70.9	33.1	60.8
Val	125.1	47.7	35.3	43.4
Leu	502.4	187.2	169.5	418.6
	Cymbella microcephala			
Ala	20.8	20.7	49.4	49.2
	Fragilaria pinnata	F = 0.173	F = 0.081	F = 0.027
all DFAA	12745.0	6888.7	6126.0	5735.9
Asp	526.8	61.4	131.9	102.3
Glu	956.7	30.5	41.8	131.7
His	4481.4	63.6	93.8	158.8
Ser	645.1	166.2	261.3	112.9
Arg/Cit	8.6	1.4	0.2	7.1
Ala	124.0	23.6	82.8	29.6
Tyr	1774.7	200.2	109.4	571.1
Phe	1606.0	121.7	236.2	283.1
Ile	426.2	4383.4	3675.6	569.5

Table 2: Continuation;

	axenic	co-culture with strain 31	co-culture with *E. coli*	co-culture with strain 32
DFAA conc. (nM)	***Pseudostaurosira sp.***			
His	3.2	19.9	948.1	927.6
	Punctastriata sp.	F = 0.797	F = 0.055	F = 0.588
all DFAA	2814.8	3452.3	11290.4	4664.7
Gly/Thr	25.7	340.4	421.5	1607.3
Met	81.6	152.9	327.9	115.5
Ile	114.1	206.2	5658.7	1364.0
	Staurosira sp.	F = 0.281	F = 0.006	F = 0.100
all DFAA	5430.1	3374.1	1586.5	2678.5
Asp	174.7	42.1	27.1	77.9
Glu	684.4	34.8	40.5	79.4
Ser	129.6	79.2	72.4	38.8
Arg/Cit	5.2	1.0	0.6	1.0
Gly/Thr	320.9	41.2	25.7	66.8
Tyr	915.1	161.8	84.5	158.8
Phe	1060.3	130.0	50.9	65.3

Cymbella microcephala: The axenic cultures reached a maximal *chl a* content of 2.27 $\mu g \cdot ml^{-1}$ after 36 days (Fig.3). Co-cultures with *Alphaproteobacterium* strain 31 showed a lower maximal *chl a* concentration (1.96 $\mu g \cdot ml^{-1}$), co-cultures with *E. coli* XL1 blue or *Bacteroidetes* strain 32 reached a higher maximal *chl a* content (up to 2.86 $\mu g \cdot ml^{-1}$) (Fig.3). The increased cell density of *C. microcephala* in co-culture with *E. coli* or strain 32 occurred in parallel to higher concentrations of alanine (Ala) in the spent culture medium at the stationary growth phase of xenic than in that of axenic cultures (Tab.1).

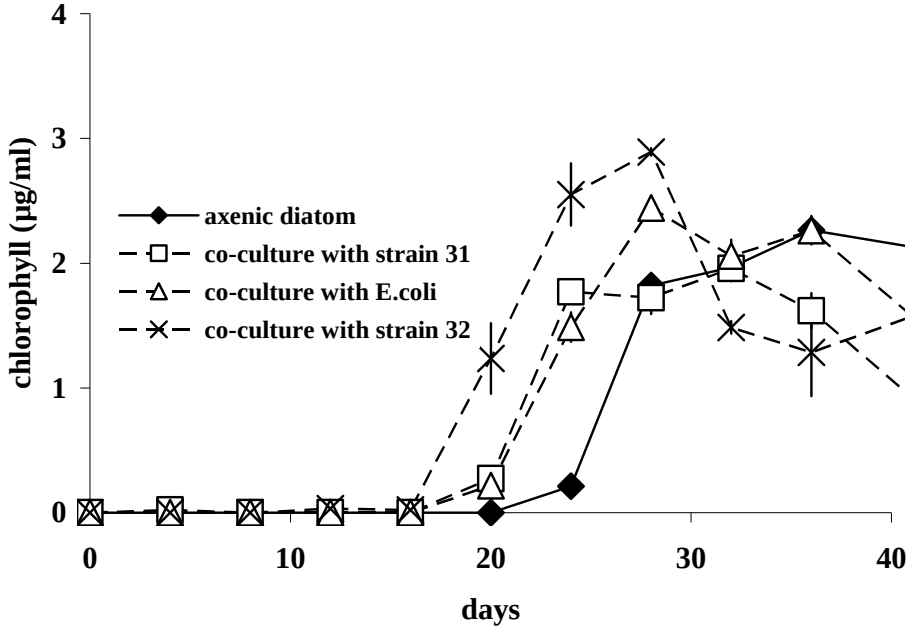

Figure 3: Growth of axenic *C. microcephala* and in co-culture with different bacteria. Various bacterial strains have different influences on the cell density of this diatom, but all enhance diatom growth rate.

Fragilaria pinnata: The axenic cultures showed a maximal content of 2.85 µg·ml^{-1} *chl a* on day 24, co-cultures with bacteria had lower maximal *chl a* concentrations of up to 2.71 µg·ml^{-1} (similar to *A. minutissima*, Fig.1). Cultures treated with 2% spent medium from XL1 blue did not grow at all whereas cultures treated with 2% spent medium from strain 32 reached maximal *chl a* concentrations (0.86 µg·ml^{-1}) after 9 days. The reduced cell density of *F. pinnata* in co-culture with bacteria was in parallel to lower DFAA concentrations in the spent culture medium at the stationary growth phase of xenic than in that of axenic cultures. Similar interrelations were found for 8 individual amino acid monomers. Reduced diatom cell density occurred together with increased concentrations of Ile in xenic than in axenic cultures (Tab.1).

Pseudostaurosira sp.: The axenic culture had a maximum *chl a* content of 1.69 µg·ml^{-1} after 20 days. Co-cultures with strains 31, 32 and XL1 blue had maximal *chl a* concentrations of 1.54 µg·ml^{-1}, 2.07 µg·ml^{-1} and 1.73 µg·ml^{-1}, respectively, diatom cell density was

influenced disparate by different bacteria (similar to *C. microcephala*, Fig.3). Cultures treated with 2% spent medium from strain 32 or XL1 blue did not grow at all. The increased cell density of *Pseudostaurosira sp.* in co-culture with *E. coli* or strain 32 was in parallel to higher concentrations of histidine (His) in the spent culture medium at the stationary growth phase of xenic than in that of axenic diatom cultures (Tab.1).

Punctastriata sp.: The axenic cultures reached a maximal *chl a* concentration of 2.02 $\mu g \cdot ml^{-1}$ after 20 days. Co-cultures with bacteria reached a higher *chl a* content (up to 2.45 $\mu g \cdot ml^{-1}$) (similar to *C. minuta*, Fig.2). Cultures treated with 2% spent medium from XL1 blue, however, did not grow at all whereas cultures treated with 2% spent medium from strain 32 reached a maximal *chl a* content of 1.66 $\mu g \cdot ml^{-1}$ already after 16 days. The higher cell density of *Punctastriata sp.* in co-culture with bacteria occurred together with higher concentrations of DFAA and 3 specific monomers in the spent culture medium at the stationary growth phase of xenic than in that of axenic cultures (Tab.2).

Staurosira sp.: All cultures reached their maximal *chl a* concentrations on day 28 whereby the axenic culture had 3.74 µg *chl a* $\cdot$ ml^{-1}, those with bacteria contained less *chl a* (up to 3.3 $\mu g \cdot ml^{-1}$) (similar to *A. minutissima*, Fig.1). Cultures treated with 2% spent medium from XL1 blue did not grow at all but cultures treated with 2% spent medium from strain 32 reached a maximal *chl a* of 0.94 $\mu g \cdot ml^{-1}$ after 28 days. The reduced cell density of *Staurosira sp.* in co-culture with bacteria was in parallel to lower concentrations of 7 amino acid monomers in the spent culture medium at the stationary growth phase of xenic than in that of axenic diatom cultures but also to lower DFAA concentrations (Tab.1).

Strain D164: Axenic cultures reached a maximal *chl a* content of 6.3 $\mu g \cdot ml^{-1}$ after 20 days of incubation. Co-cultures with bacteria reached similar concentrations.

Phaeodactylum tricornutum: The axenic cultures of Utex 646 reached a maximal *chl a* content of 17.4 $\mu g \cdot ml^{-1}$ after 24 days (Fig.4). On the same day, co-cultures with the *Alphaproteobacterium* strain 29 or the *Bacteroidetes* strain 32 reached much higher maximal

chl a concentrations of 26.9 and 27 µg·ml^{-1}, respectively. The same was true for co-cultures with XL1 blue. Bacterial effects on diatom growth were also inducible by bacterial culture supernatant. Cultures treated with 0.1% - 20% spent medium from *Alphaproteobacterium* strain 29 reached an even higher maximal *chl a* content from 22 to 28.7 µg·ml^{-1}. The more spent bacterial medium was added, the better the diatom grew. 0.01% spent medium influenced growth differently (Fig.4). Similar effects were observed with spent medium from *Bacteroidetes* strain 32 or *E. coli* XL1 blue.

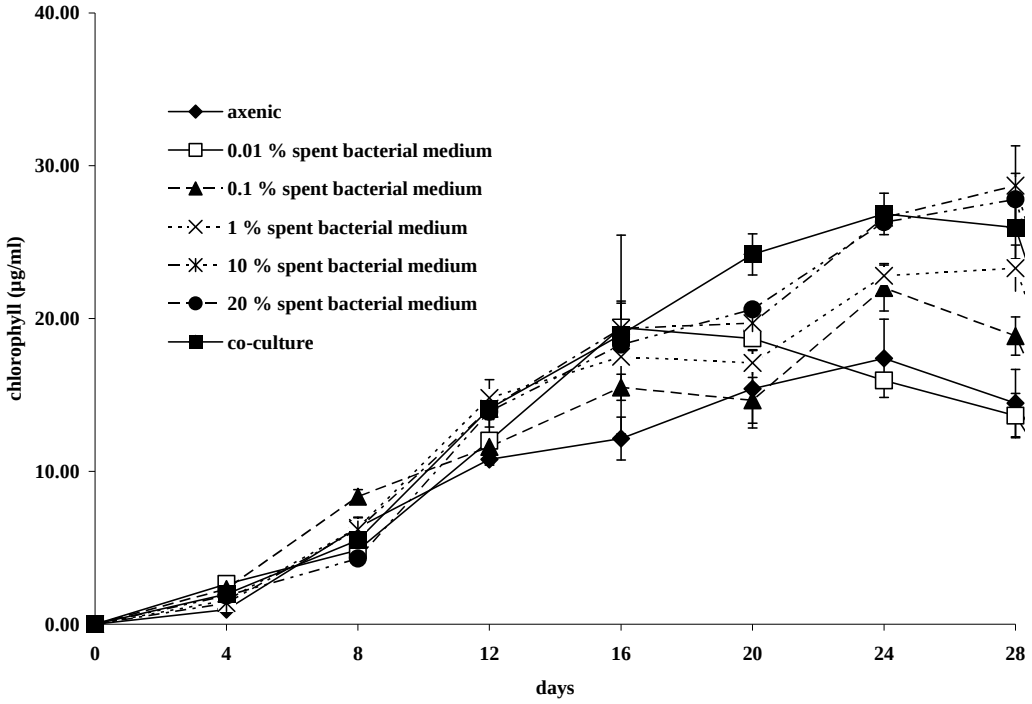

Figure 4: *P. tricornutum* Utex 646 grown in co-culture with *Alphaproteobacterium* strain 29 or its spent bacterial medium. The bacterium increases the cell density of the diatom. Applied bacterial culture supernatant increases diatom cell density already at a concentration of 0.1% (v/v). Rising concentrations leads to higher diatom cell density.

Axenic cultures of CCAP1055/1 reached a maximal *chl a* of 5.7 µg·ml^{-1} after 12 days. Co-cultures with the *Bacteroidetes* strain 32 reached higher maximal *chl a* concentrations (7.77 µg·ml^{-1}), co-cultures with K12 MG1655, however, reached lower maximal *chl a* contents of 4.7 µg·ml^{-1} after 15 days. Cultures with 2% spent medium from *Bacteroidetes*

strain 32 reached maximal *chl a* concentrations of 6.13 $\mu g \cdot ml^{-1}$ whereas cultures with spent medium from K12 MG1655 reached a maximal *chl a* content of 2.62 $\mu g \cdot ml^{-1}$.

The addition of 0.1 $g \cdot l^{-1}$ peptone inhibited diatom growth for most strains completely, some strains showed a highly decreased cell density compared to the same strain in peptone free medium.

Growth Curves and DFAA Concentration: Growth Rate

For most diatoms, measurable diatom growth in co-culture with bacteria started earlier than in axenic cultures (Fig.1 -3). For *A. minutissima* and *C. minuta* I147 this was found in parallel to decreased concentrations of DFAA or amino acid monomers in the spent culture medium at the exponential growth phase compared to the axenic strains (Tab.2). For *Punctastriata sp.* an enhanced growth rate in co-culture with bacteria correlated with higher concentrations of 2 single dissolved free amino acids. *Pseudostaurosira sp.* growth with *Proteobacteria* started earlier. This observation correlated with lower DFAA and arginine/citrulline (Arg/Cit) concentrations in the cultures. At the same time growth with *Bacteroidetes* strain 32 was retarded, while higher DFAA and Arg/Cit concentrations were detected, compared to the axenic diatom. In all other diatom/bacteria co-cultures correlations between growth rate and DFAA concentrations were more complex, involving in parallel higher and lower concentrations of single amino acid monomers compared to the axenic strains.

A generally enhanced growth rate in co-culture with bacteria was measured for *A. minutissima*, *C. minuta* (both strains), *C. microcephala*, *Punctastriata sp.*, *P. tricornutum* Utex 646 and D164, whereas *F. pinnata* and *Staurosira sp.* were generally retarded in growth. *Pseudostaurosira sp.* and *P. tricornutum* CCAP1055/1 reacted disparate to different bacteria.

Table 3: Concentrations of DFAA (nM) in the spent medium at the exponential growth phase of axenic diatom cultures and diatom bacteria co-cultures. Likelihood (F), that differences in amino acid composition between the presented DFAA pools from co-cultures compared to the axenic diatoms are not significant, was proofed by F-test.

	axenic diatom	**co-culture with strain 31**	**co-culture with *E. coli***	**co-culture with strain 32**
DFAA conc. (nM)	***Achnanthes minutissima***			
Phe	62.6	30.2	25.0	52.7
	Cymbella minuta I147	F = 0.239	F = 0.004	F = 0.031
all DFAA	5885.9	3722.1	1823.5	2566.9
His	1316.1	434.2	297.9	401.3
Ser	122.5	14.3	12.4	13.6
Arg/Cit	3.5	2.4	0.4	3.5
Gly/Thr	176.2	35.1	19.9	31.9
Ala	78.3	30.7	33.8	39.6
Tyr	659.4	241.1	60.6	332.6
Phe	703.9	93.7	66.4	227.8
Ile	583.7	361.1	187.5	271.1
	Cymbella minuta B7	F = 0.145	F = 0.013	F = 0.154
all DFAA	2108.6	1247.8	835.9	1261.8
Asp	126.5	47.4	21.0	55.2
Glu	117.4	14.0	69.1	35.2
His	520.9	207.9	122.8	165.3
Ser	56.5	29.2	22.6	28.3
Gly/Thr	96.3	26.0	0.0	18.9
Tyr	402.1	222.8	65.7	301.5
Phe	173.5	32.3	18.3	36.7
Leu	250.4	234.4	169.8	190.2
Ile	160.6	241.9	185.7	232.9
	Cymbella microcephala	F = 0.771	F = 0.475	F = 0.170
all DFAA	986.1	1125.3	1309.7	1649.7
His	84.6	169.1	296.1	193.4
Ser	0.0	27.5	46.0	27.6
Arg/Cit	1.9	2.1	2.3	3.6
Ala	0.0	25.7	20.3	48.6
Tyr	184.6	248.2	219.9	326.9
Met	30.9	57.4	54.5	67.5
Val	194.5	133.7	47.8	124.3
Phe	7.5	44.6	33.4	74.5
Ile	407.7	370.7	327.5	352.2
Leu	4.9	7.1	168.1	318.8
	Fragilaria pinnata	F = 0.603	F = 0.215	F = 0.222
His	221.8	365.2	525.5	466.9
Gly/Thr	39.4	48.7	56.9	63.6
Ala	18.2	21.2	29.0	47.7
Leu	1505.3	1121.8	641.7	702.1
	Pseudostaurosira sp.	F = 0.9	F = 0.8	F = 1.0
all DFAA	2274.1	2043.9	1685.8	2423.5
Arg/Cit	2.4	2.1	0.4	3.7
	Punctastriata sp.	F = 0.942	F = 0.812	F = 0.422
His	282.5	305.7	372.2	764.4
Ser	46.7	47.4	54.3	79.0

Table 4: Continuation;

	axenic diatom	co-culture with strain 31	co-culture with *E. coli*	co-culture with strain 32
DFAA conc. (nM)	***Staurosira sp.***	F = 0.958	F = 0.011	F = 0.976
Asp	41.0	44.7	78.2	54.0
Glu	60.1	64.5	293.0	81.0
His	260.9	661.4	1353.8	394.7
Arg/Cit	2.1	2.3	939.3	3.6
Gly/Thr	25.7	48.1	157.1	40.3
Ala	19.9	30.3	84.0	54.2
Tyr	236.5	374.9	374.1	375.1
Met	39.1	51.6	39.5	61.1
Val	104.4	88.0	63.5	79.3
Phe	60.3	114.6	357.4	93.4
Ile	287.2	26.8	103.1	137.4
Leu	683.3	318.7	253.5	487.4
	D164	F = 0.865	F = 0.678	F = 0.900
His	146.2	250.8	195.2	226.3
Ser	28.3	36.9	37.8	35.8
Gly/Thr	12.2	24.8	14.4	19.6
Ile	237.4	197.9	192.6	212.7
Leu	241.4	170.5	167.4	205.4

Bacterial effects on diatom growth rate and cell density are summarized in Tab.3, distinguishing between raphid and araphid diatoms.

Table 3: Summary of bacterial influences on diatom growth rate and cell density; the number of diatom strains is presented due to their reaction on bacterial influences regarding diatom growth;

cell density	**all diatom strains**	**raphid strains**	**araphid strains**
generally increased by bacteria	3	2	1
generally reduced by bacteria	4	2	2
dependent on bacterial strain	3	2	1
not influenced	1	1	0
growth rate			
generally enhanced by bacteria	7	6	1
generally reduced by bacteria	2	0	2
dependent on bacterial strain	2	1	1
not influenced	0	0	0

Dissolved combined Amino Acids (DCAA) from axenic Diatoms and Diatom/Bacteria Co-Cultures: Axenic cultures contained DCAA concentrations between 1.2 and 75.8 µM depending on the diatom strain. DCAA accumulated in the stationary phase in axenic and xenic cultures as well as in most co-cultures with single bacterial isolates. Some co-cultures

with bacteria showed decreased DCAA concentrations compared to those of axenic strains, e.g. *Pseudostaurosira sp*., where axenic cultures contained 75.8 µM DCAA, co-cultures with bacteria between 3.3 and 6.9 µM DCAA. The mol% composition of DCAA changed significantly with the co-cultured bacterial strains and with the growth phase of cultures (e.g. *A. minutissima*, Fig.5). Furthermore, mol% composition of DCAA was differed from that of DFAA for most cultures.

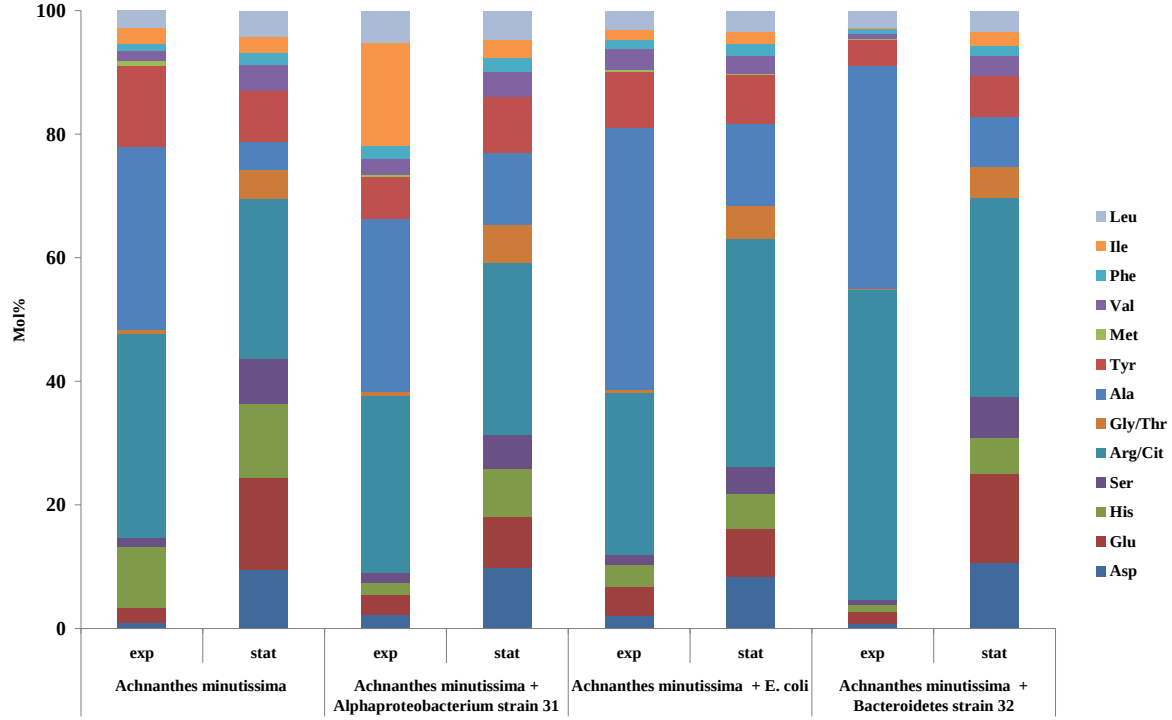

Figure 5: Proportion of different amino acids of from the dissolved combined amino acid (DCAA) pool of *A. minutissima* cultures given in mol%; DCAA contents were measured in the spent medium of the axenic diatom culture as well as of diatom/bacteria co-cultures in the exponential growth phase (exp) and the stationary growth phase (stat).

Amino Acids in Pore Water from epilithic Biofilms: Total DFAA concentration in the pore water of young biofilms (< 1 mm, ~ 6 months old) was 395 µM, in mature biofilms (> 5mm, ~ 23 months old) it was much lower and accounted for 179 µM DFAA. F-test proof, that the differences in amino acid composition between the presented DFAA pools of young and mature biofilms are significant with a likelihood of 98%. Total DCAA concentration in

samples from pore water of young biofilms was 910 µM, in mature biofilms it was lower and accounted for 593 µM DCAA.

Extracellular Carbohydrates: In most cases, we found the concentrations of soluble carbohydrates (µg carbohydrates · µg chlorophyll^{-1}) in the culture supernatant from diatom bacteria co-cultures to be different from the concentrations in axenic cultures (Tab.4). The carbohydrate monomer compositions of the co-cultures were similar to those of axenic diatoms, regarding the main monomers. The *Alphaproteobacterium* strain 31 increased the extracellular carbohydrate concentration in *A. minutissima* cultures and decreased the concentrations in co-cultures with *Staurosira* sp. The carbohydrate contents in all other diatom co-cultures with this bacterium were influenced differently at different sampling times, except for co-cultures with *C. minuta* and *C. microcephala*, where no clear differences in carbohydrate concentrations were measured compared to the axenic diatoms (Tab.4). The presence of *E. coli* resulted in an increase of the carbohydrate concentration in co-cultures with *A. minutissima*, *C. minuta* I147 and *Punctastriata sp.*. A decrease in carbohydrate concentration was found for *E. coli* co-cultures with *C. microcephala* and D164. The carbohydrate contents of all other cultures were influenced differently by *E. coli* at different sampling times (Tab.4). *Bacteroidetes* strain 32 decreased the carbohydrate concentration in co-cultures with *C. microcephala*, *C. minuta* (both strains), *F. pinnata* and *Staurosira sp.* and increased the concentration in co-cultures with *A. minutissima*. The carbohydrate contents of all other cultures were influenced disparate by strain 32 at different sampling times (Tab.4).

Table 4: Carbohydrate content (µg carbohydrates · µg chlorophyll^{-1}) of axenic epilithic diatoms and diatom bacteria co-cultures detected in the spent culture media;

diatom strains	axenic	Co-culture with *Alphaproteobacteria*	Co-culture with *E. coli*	Co-culture with *Bacteroidetes* strain 32
***A. minutissima* day 16**	not detectable	15.7±1.7	10.4±1.1	81±1.6
***A. minutissima* day 28**	53±5	93.3±8.4	120.2±4.8	163.7±17.4
***C. microcephala* day 16**	not detectable	not detectable	not detectable	not detectable
***C. microcephala* day 28**	62.7±2.3	61.7±8.4	35.5±2.6	49.1±1.3
***C. minuta* str.1 day 16**	26.7±2.6	20.3±0.4	11.6±1.1	28±0.7
***C. minuta* str.1 day 28**	29.8±5	37.2±2.1	80.2±3.2	25.9±4.4
***C. minuta* str.2 day 28**	not detectable	not detectable	not detectable	not detectable
***C. minuta* str.2 day 28**	54±2.6	50.6±2.7	91.9±15.4	27.1±2.7
***F. pinnata* day 16**	59.3±3.3	57.2±3.1	23.6±0	54.5
***F. pinnata* day 28**	38.4±7.4	24.3±2	32.2±2.4	11.5±2.4
***Staurosira* sp. day 16**	12.3±3.5	6.8±2.3	17.8±0.6	5.7±0.5
***Staurosira* sp. day 28**	43.5±8.2	40.8±2.3	17.9±1.6	11.8±2.4
***Pseudostaurosira* sp. day 16**	27.7±0.1	109.2±5.9	9.6±2.7	313.7±18.8
***Pseudostaurosira* sp. day 28**	33.2±6.5	22.5±4.2	48.3±6.9	23.5±2.5
***Punctastriata* sp. day 16**	45.6±1.4	55.5±3	43.4±2.5	9.6±1.2
***Punctastriata* sp. day 28**	17.2±1.6	14.3±0.8	29.6±2.3	33.8±2.2
D164 day 16	19.5±5.3	7.2±1.3	4.6±0.7	5.25±0.8
D164 day 28	10.6±0.4	18.1±0.2	8.9±0.3	22.4±1.7

Discussion

Extracellular Carbohydrates: In earlier studies we have shown via monomer mapping of extracellular diatom carbohydrates, that in (epilithic) diatom/bacteria co-cultures the diatoms clearly produce most of the carbohydrates whereas the carbohydrate fraction secreted by bacteria is negligible (Bruckner et al., 2008). This was confirmed for the tested diatoms in our study. According to carbohydrate secretion of diatoms in co-culture with bacteria we can classify diatoms into three groups: group (I) generally secretes more carbohydrates in the presence of bacteria, independent of the bacterial strain (one diatom in this study), group (II) generally secretes less carbohydrates (two diatoms in this study), group (III) reacts divers depending on the associated bacterial strain (six diatoms in this study). Thus, our study confirms ambiguous effects of bacteria on diatom carbohydrate secretion previously described in the literature (Wigglesworth-Cooksey & Cooksey, 2005, Grossart, 1999, Azam, 1998, Gawne et al., 1998) also for epilithic freshwater diatoms. Since bacteria apparently have a strong impact on carbohydrate secretion of nine representative biofilm forming diatom species, the interaction of these organisms must be regarded as a key factor in biofilm formation with potential impact on sediment stabilization (Stal & Brouwer, 2003; Wigglesworth-Cooksey et al, 2001; Decho, 2000; Sutherland & Grant, 1998). In nature, the occurrence of specific bacteria and bacterial communities might trigger biofilm development via enhancing the carbohydrate secretion of the diatoms. In fact, most of our axenic diatom cultures did not form biofilms; the only exception was strain D164. For planktonic organisms the interaction between diatoms and heterotrophic bacteria was already shown to be a key factor for aggregate formation (Grossart et al., 2006).

Growth: Regarding cell density of diatoms in co-culture with bacteria we can classify diatoms into four groups: group (A) generally grows denser in the presence of bacteria, independent of the bacterial strain, group (B) diatoms, which generally grow less dense in the presence of bacteria, group (C) diatoms, where cell density is highly variable in co-culture with bacteria, depending on the respective bacterial strain, and group (D) diatoms which do not show any difference in cell density by bacterial influence (one diatom in this study, D164).

Interestingly, increased cell density of the araphid (A) diatom *Punctastriata sp.* correlated with increased DFAA concentrations in co-cultures with bacteria whereas the increased cell density of the raphid (A) diatom *C. minuta* B7 occurred in parallel to decreased DFAA contents in the spent media of theses cultures.

Thus we hypothesize, that some diatom species may be generally sensitive to differences in the DFAA pools. This suggestion is supported by our experiments with artificial DFAA pools strongly influencing diatom growth, and by group (B) diatoms. Except for the raphid diatom *A. minutissima,* all group (B) strains showed parallels in cell density and overall DFAA content in the spent culture media. Interestingly, the reduced cell density correlated with lower Met concentrations in cultures of raphid (B) diatoms only (*A. minutissima*, *C. minuta* I147) and lower Asp contents in araphid (B) cultures only (*F. pinnata*, *Staurosira sp.*). Furthermore, these araphid (B) diatom cultures contained less dissolved free Glu, Ser and Phe whereas the raphid (B) cultures contained less His. All group (B) diatoms showed a decreased concentration of Arg/Cit and Tyr. Co-cultures with bacteria and *C. minuta* were performed with two different strains of this diatom species. Strain B7 belonged to group (A) diatoms but strain I147 to group (B). Interestingly, only *C. minuta* B7 co-cultures showed decreased Asp and Leu concentrations when grown with bacteria, which could explain the different interactions with bacteria between two strains from a single species. When group (C) diatoms showed an increased cell density in the presence of bacteria

this was in parallel to increased concentrations of single amino acids, His and Ala for the araphid *Pseudostaurosira sp.* and the raphid *C. microcephala* strain, respectively.

Most diatoms in our study belong to group (A) and (B), with consistent patterns in cell density in the presence of different bacteria. Our findings suggest that most diatoms react to the overall DFAA concentrations or even to specific certain DFAA monomers present in biofilms. Another important hypothetical explanation for the found phenomena could be an unknown bacterial factor causing the diatom to change actively the quality and quantity of released DFAA. Passive DFAA release by the diatoms during the interactions with bacteria is discussed below.

Comparison of DFAA concentrations in cultures in the stationary growth phase to those in the exponential growth phase revealed different possible scenarios. DFAA may be produced by the diatoms either by leakage from death cells or by secretion (Hammer & Brockmann, 1983; Poulet & Martin-Jézéquel, 1983) and cause auto inhibition of the diatom, when not degraded by bacteria (e.g. *C. minuta* I147). This hypothesis is supported by the measured high overall DFAA concentrations (~ mM) in young natural diatom dominated epilithic biofilms, whereas old biofilms contained much lower concentrations, indicating high bacterial DFAA uptake. Changed DFAA secretion by the diatoms as a reaction to bacteria could be a further explanation. DFAA concentrations in our laboratory biofilms (nM) are assumed to be higher (µM) as the presented values as well, because soluble substances were diluted by 50 ml of cultivation medium when we suspended our cells (10 - 100 mg fresh biomass) and thus released the DFAA that were trapped in the biofilm matrix. Mature biofilms, as mentioned before, contain less than half the DFAA concentration of young biofilms, where nearly mM concentrations were found by measuring only 15 single amino acids. Increasing the DFAA concentrations artificially by adding trypsin digested casein-peptone to diatom cultures often resulted in reduced diatom growth and support the above

given notion. Utilization The utilization of DFAA by diatoms as a nitrogen source under nitrogen limiting conditions has been discussed controversially (Flynn and Butler, 1986).

Another phenomenon we observed was, that the presence of much higher DFAA concentrations in diatom/bacteria co-cultures occurred together with increased diatom cell density (e.g. His in *Pseudostaurosira sp.* co-cultures with *E. coli* or strain 32). These amino acids should not derive from diatom secretions, because otherwise axenic cultures should have similar concentrations. Leakage from diatoms by bacterial degradation may be a possible explanation. The release of amino acids by bacterial degradation of extracellular diatom proteins may be questionable as a further explanation, because the monomer composition of DCAA, which depends on the co-cultivated bacterium and the respective diatom growth phase, was not reflected by the present DFAA pool, although we cannot rule out this possibility, since DCAA pools represent partially the extracellular community proteome, including different extracellular bacterial proteins, and bacteria might degrade selectively certain amino acids. Furthermore, for most co-cultures DCAA concentrations in the stationary phase are higher than in axenic cultures, indicating that protein accumulates and does not disappear. This could be due to bacterial ectohydrolases beside other proteins. Thus we suggest that bacteria may be able to secrete amino acids to control diatom growth, beside passive release from protein degradation. Of course an unknown bacterial factor influencing diatom DFAA secretion could be hypothesized here as well.

Regarding cell density most differences in monomer composition of DFAA pools from co-cultures compared to axenic diatoms were statistically highly significant, suggesting a strong influence of DFAA in a microbial micro-environment. Possibly amino acids may function as a kind of signal molecule to regulate diatom growth in biofilms. This may not be true for planktonic species, because here extracellular products can be quickly diluted by diffusion.

Regarding growth rates, all diatoms reacted to the presence of bacteria. Thus diatoms can be classified in three groups: group (1) grows generally faster in the presence of bacteria, independent of the bacterial strain (seven diatoms in this study), group (2) grows generally slower (two diatoms in this study, araphid strains only), group (3) reacts divers depending on the associated bacterial strain (two diatoms in this study).

Interestingly all group (1) diatoms were raphid, except *Punctastriata sp*. Thus, the presence of bacteria might generally favor raphid diatoms to settle on new substrates rapidly. Nevertheless, araphid species can be enhanced in their growth rate by certain bacterial strains as well. Regarding growth rate enhancement of these diatoms by bacteria, no such clear overall interrelations with DFAA concentrations were found as for their cell density. Often the found correlations between DFAA pools related to growth rate were statistically not significant. Solely for both *C. minuta* strains, decreased His, Ser, Gly/Thr, Tyr and Phe concentrations in co-culture with bacteria were in parallel to faster diatom growth whereas changing Arg/Cit concentrations reflected faster and slower *Pseudostaurosira sp.* growth.

Cross-feeding between heterotrophic and autotrophic microorganisms (Ward et al., 1998) seems to be likely in our study involving the degradation and secretion of various amounts and compositions of DFAA. Of course, other soluble signals might play an important role as well, but we can rule out vitamin B12 produced by bacteria influencing the tested diatoms (Croft et al., 2005), because this co-enzyme was substituted to the diatom medium at 50 $\mu g \cdot l^{-1}$. Additionally, several effects regarding diatom growth observed in defined co-cultures of bacteria and diatoms were similar when the diatoms were treated with the respective bacterial spent medium. However, the observed phenomena in the presence of spent medium were often more distinct, e.g. growth reducing effects often were much stronger with bacterial culture supernatants (*Cymbella minuta* str1, *Fragilaria pinnata, Staurosira sp., Phaeodactylum tricornutum* str2).

P. tricornutum Utex 646 cultures reveal that increasing concentrations of bacterial spent medium result in a concentration dependent growth enhancement of the diatom. Hence it is likely that bacterial secretions involved in influencing diatom growth can be produced constitutively by the bacteria and may consist of DFAA.

Interactions between biofilm microorganisms seem to be regulated by a complicated "conversation" possibly based on many different soluble signals (Watnick & Kolter, 2000). For gram positive bacteria signaling via amino acids is discussed (Dunny & Leonard, 1997), whereas signaling via peptides was already studied in detail (Lyon & Novick, 2004). For *Agrobacterium tumefaciens* it is known that quorum sensing interacts with y-amino butyrate (Chevrot et al., 2006). For eukaryotes most studies on amino acids as an extracellular signal were performed with *Saccharomyces cervisiae* (Abdel-Sater et al., 2004 a/b; Gaber et al., 2003, Iraqui et al., 1999). Regarding DFAA concentrations in our study bacteria are able to quickly adapt to their environment and to modify the DFAA pool to "control" their autotrophic partners according to their needs. In biofilms perceived as "city of microbes", bacteria may be regarded as diatom gardeners, more or less actively or passively, following the suggestions of Watnick & Kolter (2000) which fascinatingly parallelized microbial interactions in biofilms with human organization principles. Via degradation or release of amino acids heterotrophic bacteria may strongly contribute to the success and distribution of diatoms in biofilms and thus shaping the microbial flora.

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Analysis of the extracellular Metaproteome of Diatoms and Bacteria in Co-Cultures indicate characteristic functional Interactions

Chapter 4

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Key words: diatoms, bacteria, interaction, biofilm, extracellular proteome

Abstract

Extracellular polymeric substances (EPS) are a major component of epilithic freshwater biofilms dominated by diatoms. These EPS are thought to play an important role for the interactions between diatoms and heterotrophic bacteria. By adding spent bacterial medium to diatoms, we found diatom EPS secretion to be influenced quantitatively. By separating extracellular proteins via SDS-PAGE from diatoms and bacteria from different culture combinations we found, that bacteria or constitutively produced bacterial substances can induce or inhibit diatom protein secretion. Using the model organisms *Phaeodactylum tricornutum* Bohlin and *Escherichia coli*, the extracellular metaproteome of these organisms was characterized by mass spectrometric peptide mapping. By the assumed function of the identified proteins we discuss their potential role during biofilm formation, involving interactions of these organisms. Signaling, cell/cell aggregation, extracellular carbohydrate modification and uptake, protein and amino acid modification and osmotic stress are thought to occur within diatom bacteria aggregates.

Introduction

Biofilms. Until today most studies on microorganisms are done in pure cultures (Battin et al., 2007), but in nature most microbes are not axenic, rather grow together with various other microbes, predominantly as biofilms (Watnick & Kolter, 2000). Covering most surfaces on this planet (Kolter & Greenberg, 2006) biofilms can be regarded as an important lifestyle on earth. Biofilms are very complex communities often exhibiting a high phenotypic plurality regarding substrate and nutrient utilization, production of EPS and cell-cell communication

(e.g. quorum sensing), resulting in surprisingly coordinated multicellular behavior, even perceived as "city of microbes" (Watnick & Kolter, 2000).

Living in a biofilm can be advantageous, e.g. biofilm cells can be more resistant to changes in salinity and temperature, UV radiation, desiccation or toxins and detergents (Decho, 2000; Costeron et al., 1987). In epilithic biofilms there are interactions between primary producers (algae and cyanobacteria), bacteria, fungi, protozoa, insects, larvae, shellfish etc. (Makk et al., 2003). Single strains or even individual cells are thought to fill distinct niches within the biofilm (Bruckner et al., 2008), possibly regulated by a complicated "conversation" based on many different soluble signals (Watnick & Kolter, 2000), thus forming a metacommunity (Battin et al., 2007). In Lake Constance biofilms are widely distributed, occupying many square kilometers as we observed. It is assumed that biofilm formation is initiated by the adsorption of organic and inorganic ions to a surface, followed by the settlement of bacteria which again serve as a substrate for the attachment of eukaryotic algae (Battin et al., 2003). Such an obligatory order for substrate adhesion is discussed controversially (Cooksey & Wigglesworth-Cooksey, 1995). For Lake Constance, a large mesotrophic, warm-monomictic lake in central Europe, phototrophic organisms in biofilms on stones, sand, mud and other particles in the littoral zone, are dominated by diatoms (Bahulikar, R, unpublished data).

Extracellular polymeric Substances (EPS). EPS are main components of the biofilm matrix. They are thought to act as a kind of glue sticking particles together, thus stabilizing whole sediments (Stal & Brouwer, 2003; Wigglesworth-Cooksey et al, 2001; Decho, 2000; Sutherland & Grant, 1998). Diatom and bacteria EPS in such biofilms consist mainly of polysaccharides and proteins (Chiovitti et al., 2003). Parts of the EPS are soluble, other parts are colloidal to solid. The polymer chemistry and the surface properties of EPS are thought to play an important role for aggregate formation (Bhaskar et al., 2005). EPS may be secreted by individual diatom species for different reasons. Some raphid diatoms secrete polysaccharides

and glycoproteins for movement (Graham & Wilcox, 2000; Pickett-Heaps, 1991), others secrete pseudo filamentous tubes or capsules, again others form cell aggregates, capsules, stalks, etc. for attachment to the substrate (Hoagland et al., 1993), and thus all contribute to biofilm development and maturation. Diatom attachment is thought to be an active process that requires glycoprotein synthesis and metabolic energy (Dugdale et al., 2006; Chiovitti et al., 2003, Cooksey & Wigglesworth-Cooksey, 1995).

Interactions between Diatoms and Bacteria. In mixed biofilm communities diatom EPS is thought to interact specifically with bacterial EPS, e.g. to form colloidal aggregates (Gawne at al., 1998). Diatoms are known to be associated with extracellular bacteria, usually in close spatial contact. Relevant cross-feeding or chemical interactions between diatoms and bacteria can occur only within a certain distance. Thus a zone around algal cells within which microorganisms are influenced by algal products was defined as “phycosphere”, as an analogy to the rhizosphere of higher plants (Bell & Mitchell, 1972). Interaction of phytoplankton and bacteria is thought to be based on the degradation and cycling of organic matter produced by the phytoplankton (Grossart et al., 2005; Schäfer et al., 2002). Co-cultivation of diatoms and extracellular bacteria reveals various other effects: bacterial growth and DNA synthesis may be stimulated (Murray et al., 1986) and in parallel diatom growth may be suppressed or enhanced (Fukami et al., 1997; Hirayama & Hirayama, 1997; Baker & Herson, 1978; Ukeles, R. & Bishop, J., 1975). The adhesion strength of diatoms may be reduced (Wigglesworth-Cooksey & Cooksey, 2005), enhanced (Grossart, 1999) or even fluctuate depending on the cultivation parameters (Gawne et al., 1998). The involvement of extracellular bacterial factors is likely (Baker & Herson, 1978), accompanied by a qualitative change in biofilm-EPS composition (Grossart, 1999; Wigglesworth-Cooksey & Cooksey, 2005), which indicates that the interactions between diatoms and heterotrophic bacteria are a key factor for aggregate formation (Grossart et al., 2006; Bruckner et al., 2008).

Only few studies were done to investigate algae/bacteria interactions on a functional genetic or proteomic level or to find extracellular factors that are exchanged between these organisms. Molecular analyses and cultivation experiments show, that many algae cannot synthesize vitamin B12 und thus may get this co-enzyme from bacteria (Croft et al, 2005). In this study we grew diatoms and bacteria in defined co-cultures and added spent bacterial medium to diatoms, to test the hypothesis that permanently produced extracellular bacterial molecules influence quantity and quality of diatom EPS secretion. By extracellular metaproteome analysis from *P. tricornutum* Utex 646 and *E. coli* K12 MG1655 we demonstrate functional induction of proteins by interactions of both organisms.

Materials and Methods

Cultures. Benthic freshwater diatoms derived from biofilms from the littoral zone of Lake Constance (Bruckner et al., 2008; Medlin et al., 2008; Bahulikar et al., 2007). The *Bacteroidetes* strain 32 was isolated and purified from a xenic *Cymbella microcephala* Grunow culture (Bruckner et al., 2008). Diatom cultures were grown in diatom medium (DM) (Watanabe, 2005) with 16 h light (30 to 60 µE) using 58 W TLD PHILLIPS neon lamps followed by eight hours darkness at 16° C. The diatoms *Achnanthes minutissima* Kützing, *C. microcephala*, *Cymbella minuta* Hilse ex Rabenhorst (two strains), *Gomphonema clavatum* Ehrenberg, *Pseudostaurosira sp.* D.M. Williams & F.E. Round (two strains), *Punctastriata sp.* D. M. Williams & F. E. Round, *Staurosira sp.* C. G. Ehrenberg, the unidentified diatom strains A2, D164, E4 and I1 were grown axenic, with and without 2% (v/v) bacterial culture supernatant from the *Bacteroidetes* strain 32 and in co-culture with the respective bacterium. *P. tricornutum* was grown the same way as the epilithic diatoms, but DM was substituted with 1.6% (w/v) sea salt. Defined *P. tricornutum*/*E. coli* co-cultures and diatoms treated with

culture supernatant from *Escherichia coli* K12 MG1655 were used for this study. The growth parameters were identical as above at a temperature of 23° C. To avoid biofilm induction due to simple sedimentation, but to trigger aggregation by the interaction of both organisms, the cultures were grown in shaking flasks. Bacteria were grown in LB or half strength LB. To harvest spent bacterial medium cells were centrifuged and the supernatant was sterilized using syringe tip filters of 0.2 µm pore size.

Purification of EPS. After two weeks of cultivation, cells were centrifuged at 5625 *g* for 10 min at 4° C. The supernatants were concentrated 10 fold via a vacuum rotary evaporator and then mixed with 4 volumes of -20° C cold acetone to precipitate polymers. This mixture was kept at least for 1 h at -20° C before the samples were centrifuged for 20 min at 5625 *g* and 4° C. The resulting pellet was washed with 70% ethanol, centrifuged again as described and then dried in a clean bench. The dry mass of the cleaned polymers was quantified with a micro-scale (KERN ARJ 2R0-4M). As a reference polymers from the pure medium were used.

Proteins. Proteins precipitated from the culture supernatant were separated by sodium dodecyl sulfate poly-acrylamide (10% and 12%) gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Proteins from the pure diatom and bacteria cultures were used as controls from the spent medium as well as from cell extracts. The EPS pellets were denaturized for 10 min at 95° C in a sample buffer containing 125 mM trishydroxymethylaminomethane (Tris) -HCl (pH 8), 10% SDS, 25% glycerin, 0,025% bromophenol blue and 25% 2-mercaptoethanol. Gels were stained either by the Alphalyse silver staining protocol (http://www.pick-n-post.com/files/Silver%20staining%20protocol.pdf) or after Heukeshoven and Dernick (1986). From gels containing *P. tricornutun* and *E. coli* proteins, bands were excised for protein-identification, performed either by Alphalyse (Odense, Denmark) or by the Institute of general Botany in Jena (Germany), fulfilling standard filter criteria. Every protein was defined as identified by at least two matching peptides or by single peptide matches which were

confirmed by samples from at least two independent experimental cultivation approaches. Proteins were subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry peptide mass fingerprinting and identified by matches across the peptide sequences (Shevchenko et al. 1996), either in the National Center for Biotechnology Information database or in a *P. tricornutum*/*E. coli* database originated from whole genome sequences of these organisms (Blattner et al., 1997; http://genome.jgi-psf.org/Phatr2/Phatr2.home.html). Database analysis was performed at the servers of the DOE Joint Genome Institute, the database of Comprehensive Microbial Resources at the J. Craig Venter Institute and the Expert Protein Analysis System proteomics server of the Swiss Institute of Bioinformatics. The identified proteins were screened for secretory signals at the SignalP 3.0 Server (Bendtsen et al., 2004; Nielsen & Krogh, 1998; Nielsen et al., 1997) at the Center for Biological Sequence Analysis (CBS).

Results

Quantity of extracellular Polymers. For 12 of the 14 tested diatom strains the quantity of secreted polymers was directly influenced by the bacterial culture supernatant in a range of more than 10% difference to the control cultures (Tab.1). Cultures of *G. clavatum* and strain A2 grown with bacterial spent medium contained the same amounts of soluble EPS as the pure cultures. Cultures of *A. minutissima*, *C. microcephala*, *C. minuta* strain 1, *Pseudostaurosira sp.* strain 1 and strain 2, strain D164, strain E4 and *P. tricornutum* showed an increased amount of soluble EPS in the presence of bacterial culture supernatant while cultures of *C. minuta* strain 2, *Punctastriata sp*. and *Staurosira sp.* showed a decreased amount.

Table 1: Content of soluble polymers (µg EPS · µg chlorophyll^{-1}) from axenic diatom cultures, grown either in pure diatom medium or treated with 2% (v/v) bacterial culture supernatant

Diatom strains	pure culture	culture with bacterial spent medium	% difference
A. minutissima	6±0.3	28±3.2	367
C. microcephala	104±5	122±28.2	17
***C. minuta* str.1**	12±0.2	20±0.3	67
***C. minuta* str.2**	68 ± 3.8	34 ± 1.3	100
G. clavatum	202±12.7	206±18.3	2
***Pseudostaurosira sp.* str.1**	12±0.7	64±6.2	433
***Pseudostaurosira sp.* str2**	12±1.4	74±2.2	517
Punctastriata sp.	136±17.1	118±3.9	15
Staurosira sp.	116±8.6	40±3.6	190
Strain A2	118±30.6	120±22.4	2
Strain D164	4±0.8	12±1.5	200
Strain E4	80±3.5	102±2.7	28
Strain I1	96±5.7	86±5.5	12
P. tricornutum	0.1±0.1	80±8.2	-> ∞

Extracellular Proteins from epilithic Diatoms. When *G. clavatum* cells were grown with bacterial spent medium we detected six protein bands between 75 kD and 100 kD in EPS samples that were not present in the samples from the pure cultures, the cell pellets or the bacterial spent medium. Similarly *A. minutissima* EPS contained additional bands of 30 kD and 75 kD. In case of *C. minuta* strain 1 an additional protein band of 125 kD was found in the EPS of the pure culture. Cultures of D164 did not show any qualitative differences regarding extracellular proteins.

Extracellular Proteins from P. tricornutum/E. coli *cultures.* The soluble EPS from (i) *P. tricornutum* treated with spent medium from *E. coli*, (ii) *P. tricornutum*/*E. coli* co-cultures, (iii) axenic *P. tricornutum* and (iiii) axenic *E. coli*, revealed complex distinctive protein band patterns, hence we identified these proteins.

E. coli proteins are presented by their common names. Most *P. tricornutum* proteins do not have a common name and are presented here by their database protein identity (database Phatr2: http://genome.jgi-psf.org/Phatr2/Phatr2.home.html).

Table 2: *P. tricornutum* proteins identified from axenic cultures (a), co-cultures with *E. coli* (c) and diatom cultures treated with spent bacterial medium (t). This table is based on information from the DOE Joint Genome Institute as at November 2008;

detected in:	Name	subcellular location	ID	Locus	Homolog/Function
c, t	e_gw1.11.105.1	possibly secreted	13240	Phatr2/ chr_11: 681619-682686	cme:CMT075C DegP protease precursor (%id: 60) [Cyanidioschyzon merolae]
a, c, t	fgenesh1_pm.C_chr_4000036	possibly secreted	41856	Phatr2/ chr_4: 1171664-1173658	gi|60472998|gb|EAL70946.1| transketolase (%id: 51) [Dictyostelium discoideum]
c, t	e_gw1.11.15.1	-	13384	Phatr2/ chr_11: 230693-232722	gi|445137|prf||1908437A topoisomerase I (%id: 38) [Arabidopsis thaliana]
c, t	estExt_gwp_gw1.C_chr_30427	possibly secreted	18793	Phatr2/ chr_3: 1331148-1333723	gi|60471005|gb|EAL68975.1| TNF receptor-associated protein (%id: 38) [Dictyostelium discoideum]
c	estExt_fgenesh1_pg.C_chr_70356	possibly secreted	45679	Phatr2/ chr_7: 908135-910705	gi|2982444|emb|CAA18252.1| CLV1 receptor kinase like protein (%id: 23) [Arabidopsis thaliana]
c	estExt_fgenesh1_pg.C_chr_210083	possibly secreted	49202	Phatr2/ chr_21: 208293-209623	gi|66499868|ref|XP_393232.2| PREDICTED: similar to ENSANGP00000012703 (%id: 20) [Apis mellifera]
c, t	estExt_fgenesh1_pg.C_chr_100355	possibly secreted	46618	Phatr2/ chr_10: 913099-914924	gi|15789992|ref|NP_279816.1| hypothetical protein VNG0846C (%id: 8) [Halobacterium sp. NRC-1]
c, t	fgenesh1_pg.C_chr_3000431	-	33512	Phatr2/ chr_3: 1115626-1118292	gi|76662420|ref|XP_583499.2| PREDICTED: similar to Golgi autoantigen, golgin subfamily A member 4 (%id: 5) [Bos taurus]
a, c, t	estExt_fgenesh1_pg.C_chr_120275	possibly secreted	47165	Phatr2/ chr_12: 684235-685779	-

P. tricornutum *Proteins.* Protein identification by mass spectrometric peptide mapping identified nine *P. tricornutum* proteins overall. Two diatom proteins were found exclusively in diatom bacteria co-cultures. Seven extracellular diatom proteins were detected in cultures induced by bacterial culture supernatant as well as in co-cultures. Two of these proteins were also detected in the axenic diatom cultures. Six of these nine diatom proteins contained a possible signal sequence for secretion and/or a membrane anchor (Tab.2) detected with a likelihood of more than 87%, and one further protein (Phatr2 ID: 18793) detected with a weak likelihood of 34%. Most *P. tricornutum* proteins had no or low homologies to proteins from other organisms already investigated in their function (Phatr2 IDs: 18793, 33512, 46618, 47165 and 49202). One protein was similar to a protease by 60% (Phatr2 ID: 13240), another to a transketolase by 51%, (Phatr2 ID: 41856), two proteins were by 38% homolog to a topoisomerase (Phatr2 ID: 13384) and a tumor necrosis factor receptor-associated protein (Phatr2 ID: 18793), one protein was by 23% similar to a receptor kinase like protein (Phatr2 ID: 45679).

E. coli *Proteins.* Overall we identified 19 *E. coli* proteins in defined co-cultures. For all these proteins comprehensive information about localization and function is available from literature. Common knowledge about these proteins based on information from the database of Comprehensive Microbial Resources at the J. Craig Venter Institute as at November 2008 is shortly summarized in Tab.3. Information related to biofilm formation and cell/cell communication is presented in detail in the discussion.

Two *E. coli* proteins (glutamate decarboxylases (DceA/DceB)) were so similar to each other, that differentiation by peptide mapping was not possible. 15 of these proteins are known to be expressed membrane associated or extracellular; one protein (malate dehydrogenase Mdh) contains a possible signal sequence for secretion detected with a

likelihood of 57%. One *E. coli* protein (outer membrane protein 1b (OmpC)) was also detected in *P. tricornutum* cultures treated with spent bacterial medium (Tab.3).

Table 3: *E. coli* proteins identified from co-cultures with *P. tricornutum* (c) and diatom cultures treated with spent bacterial medium (t). In the section homolog/function functions knowledge about general functions of the proteins is shortly summarized, considering that more features exist. This table is based on information from the database of Comprehensive Microbial Resources at the J. Craig Venter Institute as at November 2008;

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detected in:	Name	subcellular location	ID	Locus	Homolog/Function
c	P39180 AG43	Cell outer membrane	P39180	b2000, JW1982	Controls colony form variation and autoaggregation; may function as an adhesion;
c	P0ABK5 CYSK	-	P0ABK5	b2414, JW2407	O^3-acetyl-L-serine + H_2S = L-cysteine + acetate;
c	P69908 DCEA	-	P69908	b3517, JW3485	Converts glutamate to gamma-aminobutyrate; the gad system helps to maintain a near-neutral intracellular pH when cells are exposed to acidic conditions:
c	P69910 DCEB	Cytoplasm, Cell membrane	P69910	b1493, JW1488	Converts glutamate to gamma-aminobutyrate; the gad system helps to maintain a near-neutral intracellular pH when cells are exposed to acidic conditions:
c	P0ABT DPS	Cytoplasm, nucleoid	P0ABT2	b0812, JW0797	Protects the cell from UV and gamma irradiation, iron and copper toxicity, thermal stress and acid and base shocks. Also shows a weak catalase activity.
c	P0A6N1 EFTU	Cytoplasm, Cell membrane	P0A6N1	b3339, JW3301	May play an important regulatory role in cell growth and in bacterial response to nutrient deprivation;
c	P0A6P9 ENO	Cytoplasm, Secreted, Cell surface	P0A6P9	b2779, JW2750	catalyzes the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate; essential for the degradation of carbohydrates;
c	P04949 FLIC	Extracellular region	P04949	b1923, JW1908	subunit protein which polymerizes to form the filaments of bacterial flagella;
c	P02943 LAMB	Cell outer membrane	P02943	b4036, JW3996	Involved in the transport of maltose and maltodextrins; receptor for several bacteriophages;
c	P69776 LPP	Cell outer membrane	P69776	b1677, JW1667	Interacts with the peptidoglycan, thus contributing to the maintenance of the structural and functional integrity of the cell envelope;
c	P61889 MDH	possibly secreted	P61889	b3236, JW3205	(S)-malate + NAD^+ = oxaloacetate + NADH;
c	P21420 NMPC	Cell outer membrane	P21420	b0553	Transport and binding proteins
c	P0A910 OMPA	Cell outer membrane	P0A910	b0957, JW0940	Required for the action of colicins K and L and for the stabilization of mating aggregates; serves as a receptor for a number of phages; also acts as a porin with low permeability;
c, t	P06996 OMPC	Cell outer membrane	P06996	b2215, JW2203	Forms passive diffusion pores ;
c	P02931 OMPF	Cell outer membrane	P02931	b0929, JW0912	Forms passive diffusion pores; receptor for the bacteriophage T2;
c	P09169 OMPT	Cell outer membrane	P09169	b0565, JW0554	Protease;
c	P0A917 OMPX	Cell outer membrane	P0A917	b0814, JW0799	-
c	P0A905 SLYB	Cell outer membrane	P0A905	b1641, JW1633	Induced by low extracellular levels of magnesium via the phoQ/phoP two-component regulatory system;
c	P77717 YBAYI	Cell membrane	P77717	b0453, JW0443	protein binding

Discussion

Diatom EPS. Most diatom strains showed a changed EPS secretion when treated with bacterial spent medium. More than half of the diatom strains showed an enhanced polymer secretion, indicating that the interaction with bacteria is one of the key-factors inducing or inhibiting EPS secretion in diatoms and thus contributing to such biofilm formation. Our study suggests further, that diatom adhesion is indeed triggered by constitutively secreted bacterial molecules, because most axenic diatoms did not form biofilms, when bacterial spent medium induced such biofilm formation. Separation of extracellular proteins revealed, that not only the EPS quantity, including polysaccharides and proteins, is influenced. Bacterial substances induced or to inhibited the secretion of certain proteins by diatoms. Grossart et al., 2006, already reported on bacterial influences regarding extracellular proteinaceous particles in diatom/bacteria co-cultures. Especially during the exponential growth of diatoms extracellular protein concentrations in diatom/bacteria co-cultures were higher than in axenic cultures. Our study confirms this observation, because most *P. tricornutum* proteins were identified in samples from co-cultures of the diatom with *E. coli*, as well as from diatom cultures treated with spent *E. coli* medium.

Interestingly most of the detected bacterial proteins are known to be involved in biofilm formation in pure *E. coli* cultures. Thus we discuss their regulation here in detail.

Regulation of Protein Expression in Biofilms. The gene transcription of sessile bacteria cells is generally thought to be different to the transcript of planktonic cells (Pruzzo et al., 1996), but our experimental situation in shaking flasks did not allowed biofilm induction by simple sedimentation of cells. Thus biofilm formation was induced by functional interactions between *P. tricornutum* and *E. coli* producing clumping cell aggregates.

Most abundant *E. coli* proteins in our study can be classified as transport and protein binding proteins.

Many of the porin forming Omp proteins were found to be induced in biofilms in this study. It was shown earlier that outer membrane protein 3a (OmpA) is involved in *E. coli* biofilm formation. Deletion of OmpA e.g. caused an 80 % decrease in *E. coli* biofilm mass in various media (Barrios et al., 2005). *E. coli* cells in biofilms overexpress OmpA (Smith et al., 2007; Orme et al., 2006). Further OmpC was found to be significantly expressed in biofilms compared to planktonic cells as well (Sauer, 2003; Schembri et al., 2003; Kuchma & O'Toole, 2000; Prigent-Combaret et al., 1999), similar to outer membrane protein 1a (OmpF) and outer membrane protein 3b/protease VII (OmpT) (Sauer, 2003; Schembri et al., 2003).

Further we detected proteins involved in binding of other proteins. NmpC, an outer membrane porin protein that binds and transports other proteins, was shown to be upregulated in biofilm cells (Schembri et al., 2003). The presence of the cell surface associated protein, antigen 43 (Ag43), an outer membrane fluffing protein, similar to adhesin, and a self-recognizing autotransporter protein, stimulated formation of an initial premature biofilm and was used to create bacterial multi species biofilms via Ag43 expressing mutants (Kjaergaard et al., 2000). Expression of Ag43 greatly enhances bacterial biofilm not only in *E. coli* (Schembri et al., 2003) but also in other gram-negative bacteria (Kjaergaard et al, 2000a/b; Klemm et al., 2004). Ag43 deficient mutants were not able to develop mature biofilms (Danese et al., 2000).

The flagellin (FliC), a filament structural protein is involved in flagella development and is important for motility of *E. coli*. Nevertheless this is not contradictionary to biofilm formation since biofilm cells are sessile, because it was shown earlier, that FliC is involved in mono-species *E. coli* biofilm formation as well, thus e.g. Danese et al., 2000, state, that flagella mediated activity is required for biofilm formation. Enhanced FliC promoter activity caused enhanced motility as well as enhanced adherence to surfaces (Barrios et al., 2006).

FliC::kan strains were severely hindered in initial stages of biofilm formation (Pratt & Kolter, 2008). In mature biofilms FliC is downregulated (Kuchma & O'Toole, 2000).

Not much is known about the role of the global regulator Dps in biofilms. Usually it is thought to occur intracellular, but phage tolerant *E. coli* biofilms showed a strong expression of Dps protein in the outer membrane protein fraction (Lacqua et al., 2006) as we found in our samples. Dps was also found to be overexpressed in biofilms (Trémoulet et al., 2002).

Further we detected the Mdh and the maltose high-affinity receptor LamB, both described to be upregulated in sessile *E. coli* cells (Beloin et al., 2004; Trémoulet et al., 2002). Mdh deficient mutants showed less biofilm development as the wildtype (Beloin et al., 2004). Their possible function in biofilms is discussed later.

For many of the identified *E. coli* proteins abundant studies were performed regarding their function. Therefore we discuss them here in detail. Information presented without references is based on information from the database of Comprehensive Microbial Resources at the J. Craig Venter Institute as at November 2008;

Attachment. Atomic force microscopy revealed OmpA to be involved in a bond between *E. coli* cells and abiotic surfaces (Lower et al., 2005). Extracellular loops of OmpA are known to bind directly brain microvascular endothelial cells (Smith et al., 2007). Deletion of outer membrane protein X (OmpX) increases cell surface adhesion of fimbriated strains of *E. coli* and decreases cell surface adhesion of nonfimbriated strains (Otto & Hermansson, 2004). The protein Ag43 is known to interact with AIDA, a potent bacterial adhesin (Sherlock et al., 2004). We found all these three proteins to be induced in our *E. coli*/*P. tricornutum* biofilms, indicating that they are involved in cell/cell aggregation.

Interactions between Prokaryotes and Eukaryotes. Extracellular loops of OmpA are known to interact with brain microvascular endothelial cells (Prasadarao et al., 1996) and to be important in invading colonic epithelial cells (Meier et al., 1996). In macrophages it induces an antiapoptotic factor and it suppresses the expression of chemokines and cytokines

in monocytes. Further on it is thought to be absolute critical in adherence to plant surfaces, because OmpA mutants are not able to colonize alfalfa sprouts (Smith et al., 2007). Sherlock et al., 2006, showed that Ag43 can bind to human cell lines. The detection of these two proteins in our biofilms containing a prokaryote and a eukaryote extends the above given assumption of their involvement in cell/cell attachment to a direct involvement in cell aggregation between *P. tricornutum* and *E. coli*

The protein chain elongation factor EfTu is known as a very abundant protein in *E. coli*. Nevertheless it seems to be involved in communication with plants. Zipfel et al, 2006, showed that *Arabidopsis thaliana* detects EfTu and answers by complex signal cascades.

P. tricornutum as a diatom is a plant as well and in addition shows further attributes of mammalian and oomycete cells (Scala et al., 2002), thus a direct involvement of OmpA, Ag43 and EfTu in the interaction between the diatom and the bacterium is likely, possibly related to signaling events.

Signaling. Interestingly, the expression of OmpX as well as Dps responds to a global signal, acetyl phosphate, which functions during biofilm development (Wolfe et al., 2003, Trémoulet et al., 2002). EfTu was described to be involved in communication with plants (Zipfel et al., 2006). These three *E. coli* proteins indicate that signaling happened in our biofilms, not only for the prokaryotes themselves, but between the bacterium and the diatom as well.

Identified diatom proteins support this hypothesis, thus the homolog to a CLV1 receptor kinase (Phatr2 ID: 45679) and the homolog to a tumor necrosis factor (TNF) receptor associated protein (Phatr2 ID: 18793). The CLV1 kinase of *A. thaliana* is important for the organized development and proliferation of shoot and floral meristems and senses extracellular signals similar to animal hormone receptors (Clark et al., 1997). TNF receptors from mammals are beside other functions known to be involved in a complicated

"conversation" between mammalian cells and bacteria. Thereby bacterial DNA, lipopolysaccharide and peptidoglycan are involved as well.

EPS and Monomer Secretion, Modification and Uptake. As mentioned above, EPS are the main components of the biofilm matrix, consisting predominantly of polysaccharides and proteins in biofilms dominated by diatoms (Chiovitti et al., 2003). As an extracellular *P. tricornutum* protein we identified a homologue to a protease (Phatr2 ID: 13240) in *E. coli* modified cultures, indicating that extracellular protein degradation is important in diatom bacteria biofilms, as postulated by Grossart et al., 2006. A transketolase homolog (Phatr2 ID: 41856) derived from the diatom indicates the extracellular modification of carbohydrates by diatoms; it was detected in the axenic culture as well and indicates that diatoms modify their own extracellular carbohydrates. However, an extracellular diatom transketolase activity was never described in literature, but contamination by intracellular protein can be excluded, because we did not identified proteins from typical diatom housekeeping genes in our samples. In literature, even pathways for fixation of inorganic carbon are discussed to be localized extracellular (Puscaric & Mortain-Bertrand, 2003).

The bacterial degradation of diatom polysaccharides as described earlier (Bruckner et al., 2008) and its subsequent utilization as a substrate is likely. We speculate that Ybay could be involved in polysaccharide degradation, as it is thought to play a role in glycoprotein/polysaccharide modification by its structural features. Released sugar monomers or oligomers can be addressed to the detected LamB which is known to be involved in carbohydrate transport. As a carbohydrate degrading *E. coli* enzyme, we detected enolase (Eno) extracellulary, which is known to be essential for carbohydrate degradation. As a further bacterial enzyme involved in carbohydrate degradation Mdh was detected, converting malate to oxalacetate. This protein is thought to occur intracellular, but nevertheless it contains a possible signal for secretion.

Beside enzymes involved in carbohydrate metabolism we detected the protease OmpT and DceA/B and O-acetylserine sulfhydrolase A (CysK), enzymes involved in amino acid degradation and formation. Thereby the isoform DceB is known to be associated with the cell membrane. Extracellular enzyme activity regarding degradation of polysaccharides and proteins in mixed diatom bacteria biofilms was already well described (Azam, 1998). Interestingly we found homologues to such proteins for the diatom as well.

Osmotic Stress, pH Shifts and Ion Deprivation: In biofilms EPS are thought to act as a kind of ion trap (Chin et al., 1998). Thus the cellular environment in biofilms is thought to have a high osmolarity (Prigent-Combaret et al., 1999). The detected *E. coli* protein OmpC is known to be upregulated at high osmolarity via EnvZ/OmpR, a two-component signal transduction pathway originally shown to regulate expression of the outer membrane porins OmpF and OmpC (Prüß et al., 2006). Such an enrichment of ions can lead to pH shifts. The detected Dps is known to protect *E. coli* cells from acid and base shocks, as well as DceB. Further we detected the structural murein lipoprotein Lpp in our extracellular protein samples from biofilms. Lpp is known to be upregulated during envelope stress induced *E. coli* biofilm formation (Yang et al., 2008), indicating that *E. coli* has to maintain its envelope integrity. This may be related to osmotic stress.

Another outer membrane protein, SlyB, thought to be induced in *E. coli* by magnesium deficiency, was detected, indicating a competition for inorganic ions between the diatom and the bacterium.

Conclusively we want to summarize that we provided new insights in diatom/bacteria interactions by our metaproteome experiments. We can clearly show that protein secretion is regulated during these interactions. Surprisingly the identified proteins indicate comprehensible functions in these biofilms and thus provide a new and broad basis to study these interactions in detail.

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General Discussion

General Discussion

Reflecting the functional knowledge about the extracellular proteins we detected during biofilm formation in the extracellular metaproteome of *Phaeodactylum tricornutum* Bohlin and *Escherichia coli* co-cultures (chapter 4) we address the interactions between diatoms and bacteria to involve the following features:

(1) Attachment, either to the substratum or to form cell/cell aggregates;
(2) Secretion of extracellular polymeric substances and monomers, modification and uptake;
(3) Direct chemical interactions between diatoms and bacteria that can range from processes like signaling to mucus degradation;
(4) Osmotic shifts, pH shifts and ion deprivation;

The processes (1) to (3) were studied intensively and are summarized and discussed in the following paragraphs in detail.

Attachment and Adaptation to Micro-Niches: Microscopic observations revealed that mostly *Alphaproteobacteria* and *Bacteroidetes* are directly attached to diatom cells or the surrounding EPS. Bacterial proteins thought to be involved in such direct attachment were detected in the extracellular diatom/bacteria metaproteome (chapter 4). Phylogenetic analyses of bacteria associated with diatoms from different genera exhibited striking similarities of their 16S rRNA gene sequences (chapter 2). Especially *Alphaproteobacteria* were the dominating bacteria as reported earlier (Grossart et al., 2005; Riemann et al., 2000), but also *Gammaproteobacteria* and *Bacteroidetes* were found in diatom cultures.

The latter are known to be frequently associated with diatoms (Grossart et al., 2005; Knoll et al., 2001; Riemann et al., 2000). Interestingly, the abundant types of 16S rRNA gene sequences derived from our samples have recent common ancestors and belonged to *Alphaproteobacteria* and *Bacteroidetes*, although the tested diatoms included raphid and araphid species and were phylogenetically highly diverse. Since literature from earlier studies confirm close phylogenetic relationships between bacteria from the above mentioned groups, diatoms generally are addressed to provide a micro-environment to which especially *Proteobacteria* and *Bacteroidetes* have adapted and evolved separately. Diatom samples, where such close phylogenetic bacterial relationships were observed earlier, derived from planktonic and/or benthic diatoms from Scripps Pier (San Diego, California) (Riemann et al., 2000), the Gulf of Mexico and Amityville (New York) (Schäfer et al., 2002), the Nordsee (Germany) (Grossart et al., 2005), the arctic ice (Bowman et al., 1997) or the Danube (Makk et al., 2003).

Purification of diatoms from the associated bacterial community indicated already, that interaction between diatoms and bacteria are complicated, revealing different grades of mutual interdependencies. For instance bacteria were observed in diatom cultures microscopically, but cultivation of these bacteria on standard bacteria media was not successful (chapter 1). For most diatom cultures individual purification procedures had to be developed. Although some procedures were described in chapter 1 various other methods did not result in axenic diatom cultures. In our studies it was not possible to purify especially large (> 50 µM) motile pennate diatoms. Generally, applying harsh antibiotics concentrations to diatom bacteria mixtures for short periods combined with sterical separation were the most successful approaches. Then single diatom cells had to be removed and transferred in antibiotics free medium. For other diatoms an intermediate co-cultivation with *E. coli* during the purification procedure had to be applied. The success of this artificial co-culture during diatom purification indicates, that for some strains mutual dependency is not always related to

special diatom adapted bacterial strains. Moreover *E. coli* and the studied diatoms were able to interact quickly with each other for the benefit of both.

Very interesting was the fact, that most of our axenic cultures did not form biofilms, only one strain (D164) behaved differently. Beside the formation of colloidal structures in mixed biofilm communities where diatom EPS can interact specifically with bacterial EPS (Gawne at al., 1998), bacteria may release or degrade substances that are involved in biofilm induction or inhibition. Since the main components of the biofilm matrix are extracellular polymeric substances (EPS) (Chiovitti et al., 2003) a direct influence of bacteria is possible, indicating that the presence of bacteria or bacterial substances might be needed to induce EPS production of diatoms.

Secretion of extracellular Polymeric Substances and Monomers, Modification and Uptake: By analyzing the carbohydrate content and the monomer composition of the respective carbohydrates from axenic diatom cultures as well as from diatom/bacteria co-cultures (chapter 2 and 3), we found that the extracellular community carbohydrates derived predominately from the diatom. *Proteobacteria* enhanced secretion of soluble carbohydrates by *C. microcephala* whereas a *Bacteroidetes* strain or spent medium from this bacterium reduced the secretion of soluble carbohydrates by the diatom and caused in parallel accumulation of cell associated carbohydrates, indicating an unknown bacterial factor influencing the diatom secretions (chapter 2). This direct influence supports the hypothesis of adaptation of *Bacteroidetes* to diatom mats as indicated by phylogenetic studies.

C. microcephala is a representative epilithic freshwater diatom. Nevertheless we were interested in the question whether bacterial effects on carbohydrate secretion of this diatom are similar for other representative diatom strains. Thus we tried to analyse similar effects for 12 diatom strains including 9 species, and three bacterial strains. Regarding carbohydrate secretion of diatoms our study confirms ambiguous effects of bacteria on diatom as previously

described in the literature (Wigglesworth-Cooksey & Cooksey, 2005, Grossart, 1999, Azam, 1998, Gawne et al., 1998). We can classify diatoms in co-culture with bacteria into three groups, reacting either generally in their carbohydrate secretion to the presence of bacteria, independent of the bacterial strain (two groups), or reacting divers, depending on the associated bacterial strain. Most diatoms belonged to the latter group, indicating that carbohydrate secretion in natural diatom dominated biofilms strongly depends on the associated eubacterial community. In nature the occurrence of specific bacterial communities might trigger biofilm development via enhancing the carbohydrate secretion of the diatoms as indicated for aggregation of planktonic diatoms by Grossart et al. (2006). Of course, other biotic and abiotic factors strongly influence diatom carbohydrate secretion as well. E.g. diatoms secrete polysaccharides dependent on the stage of their cell cycle, often influencing characteristically cell or colony morphology, depending on the diatom species (Graham & Wilcox, 2000; Becker, 1995; Hoagland et al., 1993; Pickett-Heaps, 1991). Polysaccharide secretion is influenced further by the surrounding nutrients (Guzmán-Morillo et al., 2007; Abdullahi et al., 2006; Magaletti et al., 2004; Underwood et al., 2004).

Such polysaccharides are thought to be a carbon source for heterotrophic bacteria (Haynes et al., 2007; Giroldo et al., 2003). By comparing diatom growth to microbial community growth and carbohydrate concentrations, we found *Alphaproteobacteria* to appear to utilize cell-bound polysaccharides and *Betaproteobacteria* to appear to utilize soluble carbohydrates as their carbon source beside other substrates, thus supporting the hypothesis of adaptation of *Alphaproteobacteria* to diatom mats as indicated by phylogenetic studies (chapter 2). Interestingly proteins thought to be involved in polysaccharide and carbohydrate degradation and transportation were detected in the extracellular diatom/bacteria metaproteome, derived from bacteria as well as from diatoms (chapter 4).

Diatom EPS does not contain only polysaccharides but proteins as well. Thus we tried to answer the question, whether bacteria influence exclusively diatom carbohydrate secretion

or protein secretion as well. Interestingly 12 of 14 diatom strains showed a changed EPS secretion, including polysaccharides as well as proteins, when treated with bacterial spent medium (chapter 4). Separation of extracellular proteins revealed, that not only the EPS quantity is influenced, but bacterial substances also induced or to inhibited the secretion of certain proteins by diatoms as indicated by Grossart et al. (2006). They reported bacterial influences regarding extracellular proteinaceous particles in diatom/bacteria co-cultures. These findings further suggests, that diatom adhesion is indeed triggered by constitutively secreted bacterial molecules, because most axenic diatoms did not form biofilms, when on the other hand bacterial spent medium induced diatom biofilm formation, and diatom adhesion is known to need the production of extracellular protein (Dugdale et al., 2006; Chiovitti et al., 2003, Cooksey & Wigglesworth-Cooksey, 1995).

Direct chemical Interactions between Diatoms and Bacteria: In diatom/bacteria co-cultures with the ubiquitous freshwater diatom *C. microcephala* (chapter 2), we showed that the diatoms produced the organic carbon source for these bacteria and supported diatom growth, confirming Grossart (1999) and Fukami et al. (1997). Apparently the bacteria released substances that supported growth of *C. microcephala*, or they consumed substances that might have otherwise inhibited diatom growth. In a rather complex co-culture approach (chapter 3) we used 9 epilithic biofilm freshwater diatoms and two associated bacteria together with the model organisms *Escherichia coli* and *Phaeodactylum tricornutum* to monitor the influences of such substances. In earlier preliminary experiments we identified free dissolved amino acid (DFAA) pools to be a compound in bacterial spent medium influencing diatom growth strongly, often inhibitory. Via cultivating various diatoms with single amino acids we found the growth of diatoms to be regulated by these molecules in a complicated manner. Most diatoms reacted disparate to different amino acids by enhanced or

reduced growth rate and increased or decreased cell density. We could monitor such effects of DFAA already at amino acid concentrations of 1nM (Miriam Windler, unpublished data).

Regarding cell density of diatoms in co-culture with bacteria we can classify diatoms in four groups: reacting either generally in their cell density to the presence of bacteria, independent of the bacterial strain (two groups), or reacting divers, depending on the associated bacterial strain, or showing no reaction to the presence of bacteria (chapter 2/3).

Most of the diatoms in our experiments reacted very generally to different bacteria. Interestingly enhanced or reduced cell density of the diatoms could be related to the concentrations of DFAA, indicating that more or less specifically composed DFAA pools or single amino acid monomers influence diatom growth, or that bacteria influence diatom DFAA secretion. Regarding growth rate, all diatoms reacted to the presence of bacteria and correlations to DFAA concentrations were found as well (chapter 3).

Beside secretion by the diatom (Hammer & Brockmann, 1983; Poulet & Martin-Jézéquel, 1983) DFAA may be produced by leakage from death diatom cells and cause auto inhibition of the diatom, when not degraded by bacteria. This notion is supported by artificially applied DFAA pools often inhibiting diatom growth, and by high DFAA concentrations we detected in approximately six month old natural diatom dominated epilithic biofilms, whereas two years old biofilms contained much lower DFAA concentrations (chapter 3).

Further bacteria may have an influence on diatom growth by releasing amino acids from degrading diatom cells, extracellular diatom proteins, or by direct secretion of amino acids. If the diatoms themselves are not releasing DFAA as an answer to bacterial influences, amino acids possibly function as a kind of ubiquitous signal molecule to regulate diatom growth in biofilms and thus could be regarded as quorum sensing regulators. Biofilm microorganisms are addressed to regulate their interactions by a complicated “conversation” based on many different soluble signals (Watnick & Kolter, 2000). Additionally, due to our

experiments, it is likely that bacterial secretions involved in “communication” with the diatoms can be produced constitutively by the bacteria (chapter 3). Several effects regarding diatom growth observed in defined co-cultures of bacteria and diatoms in our studies were similar or even stronger when the diatoms were treated with the respective bacterial spent medium. We postulate that DFAA could be one of these signals involved in “communication” between diatoms and bacteria. For bacteria signalling via amino acids is discussed (Chevrot et al., 2006; Dunny & Leonard, 1997), whereas signalling via peptides was already studied in detail (Lyon & Novick, 2004). For eukaryotes most studies on amino acids as an extracellular signal were performed with yeast (Abdel-Sater et al., 2004 a/b; Gaber et al., 2003, Iraqui et al., 1999). One interesting perspective might be that via degradation or release of amino acids heterotrophic bacteria strongly contribute to the success and distribution of diatoms in biofilms, thus shape the microbial flora. This hypothesis is supported by bacterial proteins thought to be involved in protein and amino acid degradation that we detected in the extracellular diatom/bacteria metaproteome (chapter 4).

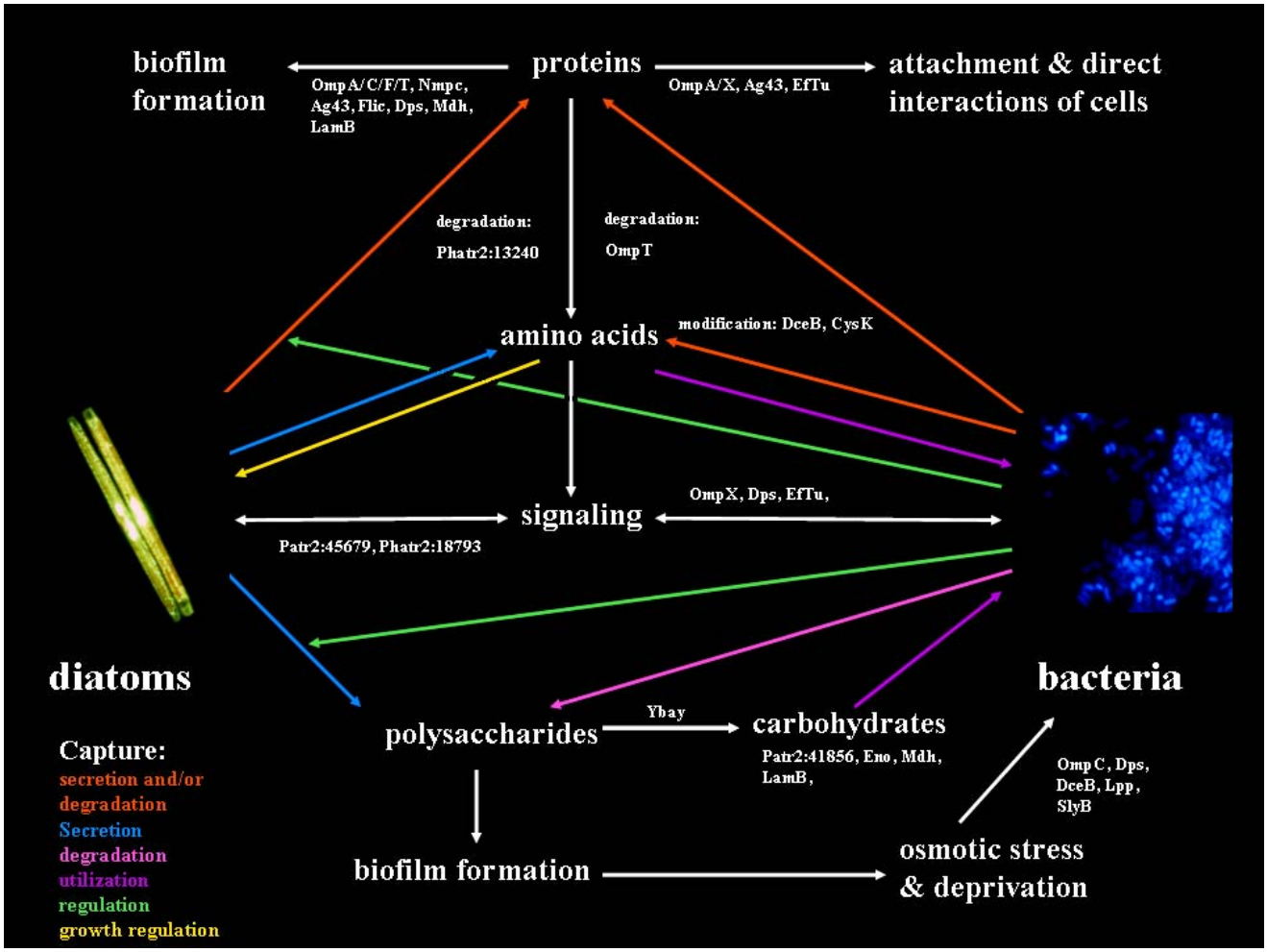

Figure 1: A hypothetical model deduced from this study for diatom/bacteria interactions in biofilms.

We want to stress finally, that natural epilithic biofilms are much more complex than the already complicated biofilms produced in the laboratories for our studies, where a single xenic diatom strain contained mathematically estimated already up to 150 different associated bacteria strains. By microscopy often up to 100 different diatom species appear in a single drop of biofilm suspension. Watnick and Colter (2000) parallelized biofilm communities with city life: “There are several steps that we must take to optimize our lives in a city. The first is to choose the city in which we will live, then we must select our neighborhood in the city that best suits our needs, and finally we must make our home amongst the homes of many others. Occasionally, when life in the city sours, we leave. The same steps occur in the formation of a […] biofilm.”

Summary

In this study we developed methods to purify diatoms from associated bacteria. Therefore most diatoms could be purified by short term harsh antibiotics treatment followed by single cell care. Diatoms that are difficult to purify could be made axenic via an intermediate defined co-culture with *Escherichia coli*. Phylogenetic analysis via 16S rRNA gene profiling of diatom associated bacteria indicated that *Alphaproteobacteria* and *Bacteroidetes* have adapted to the micro environment diatom biofilm. Defined co-cultures of diatoms and bacteria support this hypothesis by indicating that different bacteria utilize different fractions of secreted diatom carbohydrates. Bacteria were found to influence diatom growth strongly, either direct or by constitutively released soluble substances. We found concentrations of dissolved free amino acids (DFAA), analyzed by HPLC via ortho-phthaldialdehyde derivatization, to correlate with diatom growth when influenced by bacteria, and thus postulate that DFAA may be one class of such substances, regulating growth rate and growth density of diatoms. Further bacteria have a strong influence on the secretion of extracellular polymeric substances (EPS) by the diatoms. Investigating diatom carbohydrates by HPAE-PAD and spectrophotometric assays revealed that diatom carbohydrate secretion is influenced predominately quantitatively by bacteria. By quantifying diatom protein secretion and separating diatom proteins via SDS-PAGE we found diatom protein secretion to be influenced qualitatively and quantitatively by bacteria. The model organisms *Phaeodactylum tricornutum* and *Escherichia coli* were used to identify via Maldi-tof mass spectrometry peptide mass fingerprinting extracellular bacteria and diatom proteins that are induced during biofilm formation as an interaction of these organisms. These proteins could be related to different functions that are thought to play an important role during this interaction.

Zusammenfassung

Im Rahmen dieser Arbeit wurden systematisch Methoden entwickelt, um Diatomeen von assoziierten Bakterien zu reinigen. Die meisten Kieselalgen konnten dabei durch kurzzeitige Behandlung mit hochkonzentrierten Antibiotika-Kombinationen von Bakterien befreit werden, wobei in schwierigen Fällen eine intermediäre *Escherichia coli* Co-Kultur hilfreich war. Phylogenetische Studien indizieren, dass *Alphaproteobakteria* und *Bacteroidetes* sich an den Mikrolebensraum Kieselalgenbiofilm anpassen konnten. Definierte Co-Kulturen aus Diatomeen und Bakterien unterstützen diese Hypothese, da verschiedene dieser Bakterien unterschiedliche Fraktionen der von den Kieselalgen sekretierten Kohlenhydrate verstoffwechselten. Ferner konnte gezeigt werden, dass Bakterien das Wachstum der Diatomeen stark beeinflussen, entweder direkt oder über konstitutiv freigesetzte lösliche Faktoren. Mittels HPLC-Quantifizierung über Ortho-Phthaldialdehyd Derivatisierung freier löslicher Aminosäuren (DFAA) fanden wir Korrelationen zwischen DFAA Konzentrationen und Diatomeenwachstum unter bakteriellem Einfluss. DFAA könnten daher eine Substanzklasse darstellen, die einen regulativen Einfluss auf Kieselalgen ausübt und Wachstumsgeschwindigkeit und Wachstumsdichte der Diatomeen steuert. Weiterhin üben Bakterien einen starken Einfluss auf die Sekretion extrazellulärer polymerer Substanzen durch die Kieselalgen aus: mittels spektrophotometrischer Quantifizierung und HPAE-PAD fanden wir Diatomeenkohlenhydratsekretion vor allem quantitativ beeinflusst, Proteinsekretion aber quantitativ wie qualitativ. Die Modellorganismen *Phaeodactylum tricornutum* und *Escherichia coli* wurden verwendet um mittels Maldi-tof Massenspektrometrie-Peptidmassenabgleich Proteine zu identifizieren, welche bei der Biofilmbildung als Funktion der Wechselwirkung zwischen den Organismen induziert werden. Diese Proteine konnten umfassenden Funktionen zugeordnet werden und indizieren dadurch wichtige Vorgänge während der Biofilmbildung durch Kieselalgen und Bakterien.

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Contributions

All work has been carried out by me or by students under my supervision unless stated. This work has been carried out from February 2006 until December 2008, except the clone libraries from chapter 2, which were made 2005, under the guidance of Prof. Dr. Kroth. Manuscripts were corrected by Priv. Doz. Dr. Hans-Peter Grossart, Prof. Dr. Peter Kroth and Prof. Dr. Bernhard Schink.

Chapter 2: Rahul Bahulikar did half of the 16S rRNA gene clone libraries; he isolated the diatoms, extracted DNA from xenic diatom cultures and found *C. microcephala* to be dominant in Lake Constance;
Monali Rahalkar did the phylogenetic analysis of the 16S rRNA genes;
Rahul Bahulikar and Monali Rahalkar strongly contributed in writing the phylogenetic part of chapter 2

Chapter 3: Amino acid analysis was carried out by Hans-Peter Grossart or by me under his supervision; Charlotte Rehm did parts of the growth curves.

Chapter 4: Mass spectrometric peptide mapping was done by Wolfram Weisheit and Maria Mittag; Charlotte Rehm did parts of the EPS characterization of the epilithic freshwater diatoms

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