Characterisation of biotic interactions between a *Dyadobacter* strain and the diatom *Achnanthidium minutissimum*

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Abstract

Diatoms are phototrophic, unicellular algae. Due to their large share in CO₂ fixation and O₂ production, they are very important for Earth’s biogeochemistry. Many diatoms are also of economic relevance, due to their capacity to colonise submerged surfaces such as ship hulls. This so called biofouling includes the undesired growth of biofilms, which form by the aggregation of cells and the secretion of a matrix of extracellular polymeric substances (EPS). Depending on the composition of the biofilm matrix, this microenvironment of the embedded cells can be quite different compared to the surrounding environment. Naturally occurring biofilms are often complex communities of microorganisms from different kingdoms of life, such as photoautotrophic diatoms and heterotrophic bacteria. Complex intercellular interactions occur in such communities, but only few signalling or messenger molecules, which constitute these chemical communication pathways, are known. Therefore, the identification of such molecules is of great interest.

In this thesis, a biological assay system ("bioassay") was tested to study biotic interactions between the freshwater diatom *Achnanthidium minutissimum* and a biofilm-dwelling bacterium from Lake Constance. It was intended to elucidate chemical modes of communication between these species and to identify relevant molecules. An additional goal was the investigation of EPS capsule formation by the diatom in reaction to contact with the bacterium.

For these purposes, an assay that quantifies the biofilm formation of the diatom was improved and automated. The assay was also used as a reporter system for the fractionation of bioactive compounds from the bacterial supernatant. The supernatant production was upscaled by optimising the bacterium’s growth conditions. A combination of pH-dependent liquid-liquid and solid phase extraction was used for the fractionation of the supernatant. Moreover, light and electron microscopy were correlated in an easily adaptable manner to enable the microstructural analysis of EPS capsules.

The bioassay with *A. minutissimum* and the *Dyadobacter*-related Bacteroidetes strain clarified that its biofilm induction is mediated by soluble, yet hydrophobic, bacterial compounds. These could be extracted, albeit in low purities and amounts. However, reproducibility and user-friendliness of the staining-dependent bioassay could be enhanced by its partial automation. This was achieved by implementing both human- and machine-readable data structures, which improved the planning of experiments, simplified their repetition, as well as increased the efficiency of data evaluation in the context of medium-throughput screenings. Moreover, tests of biofilm quantification via staining-independent parameters allowed to further simplify the measurement of this bioassay. The scanning electron microscopy uncovered novel fibrillar microstructures both on the diatom frustules as well as in the material of the EPS capsule.

It can be postulated that this capsule forms by condensation of a mesh of frustule-attached fibrils. Due to a quantifiable, preferential attachment of bacteria cells to the capsules, their relevance for the interaction of diatom and bacteria is confirmed. The fractionation methods tested here highlight problems and necessary improvement options for the purification of bioactive substances in the *A. minutissimum* bioassay in sufficient quantities for molecular analyses. Moreover, the partial automation of measurement and data processing workflows highlight additional fields of application for this biofilm assay. The workflows developed here may also be beneficial for other biofilm assays.
Zusammenfassung


Chapter 1

General introduction

Diatoms are unicellular, phototrophic eukaryotes of the class Bacillariophyceae. Their defining features are their “spectacularly” designed cell walls [Kröger & Sumper, 1998], and their four-membraned plastids, which originate from secondary endocytobiosis [Kroth & Strotmann, 1999]. The cell walls of diatoms are scaffolded by biomineralised silica, to which polysaccharides and proteins are attached [Kröger & Sumper, 1998]. This so called frustule is split along a “girdle” region into two parts called thecae, which explains the etymology of this algae group’s name: “dia” and “temnein” together mean “cut in half” in Greek. Several diatom genera possess one or two major slits (called raphes) on one or either frustule surface (called valves). Raphes and valve pores can secrete extracellular polymeric substances (EPS). By definition, polymeric substances are characterised as extracellular, if they occur outside of the plasma membrane [Hoagland et al., 1993]. EPS are relevant for the attachment of cells to a surface, their motility, as well as for biofilm formation.

Cell division in diatoms is coupled to the transfer of one of the thecae to each daughter cell. The daughters only synthesise the smaller hypotheca, so that one lineage is continually shrinking, which leads to a sexual reproduction cycle [Geitler, 1932]. Thus, the long-term cultivation of diatoms, maintenance of those cultures, as well as life cycle studies encounter unique problems (see Chepurnov et al. [2004] for a review). Besides decreasing cell size, another such problem can be the decrease of the specific surface area and pore size of frustules, due to lower salt concentrations [Vrielings et al., 2007]. This results in a denser biosilica packing, and illustrates that diatom cells can modulate their frustule morphology through metabolic processes, which in turn may be influenced by genetic modification. It is envisioned to utilise such modified diatom frustules to engineer nanomaterials, which may be useful for chemical catalysis, particle separation and other applications [Kröger, 2007].

The frustules also help to trace diatoms through the geological record. Non-marine diatoms appear in ca. 70 million years old strata from the Late Cretaceous [Chacón-Baca et al., 2002], and likely radiated from marine species that evolved earlier in that period [Harwood & Nikolaev, 1995]. Genetic evidence points to an even earlier origin, up to 266 million years ago [Kooistra & Medlin, 1996]. Thus, the radiation of diatoms into almost all moist habitats likely started in the Late Permian, Trias or Jurassic. Since then, diatoms have occupied a diverse range of ecological niches and follow various life styles: planktonic [Kooistra et al., 2007] and benthic [Round, 1971] in sea- and fresh water, epibiotically on both plants and animals [Tiffany, 2011; Majewska et al., 2015], and also terrestrial [Souffreau et al., 2013].

Their diversity and abundance make diatoms a major biogeochemical and ecological force. Besides driving the silica cycle by incorporating orthosilicic acid into their cell walls, diatom photosynthesis contributes approx. 20% to the global net primary production and oxygen production [Field et al., 1998; Mann, 1999]. Additionally, diatoms often dominate the initial phase of phytoplankton blooms. Such blooms occur naturally due to the upwelling of nitrate-, phosphate- and iron-rich deep waters, as well as due to the influx of
CHAPTER 1. GENERAL INTRODUCTION

these nutrients from land [Capone & Hutchins, 2013]. Some phytoplankton blooms are harmful to the higher trophic levels due to the exhaustion of oxygen by heterotrophs that degrade the remains of the primary producers. Blooms can also be harmful to humans directly, due to the production of toxin by the microorganisms [Smayda, 1997]. Nonetheless, they are highly productive events in terms of CO$_2$ fixation, and export much of that as particulate organic carbon to deeper ocean layers [Buesseler, 1998], thus feeding the benthic heterotrophs. Organic exudates of marine diatoms can also become aerosolised via sea spray, and have recently been suggested as an important source for ice-nucleating particles, which play a role in cloud formation [Wilson et al., 2015]. For these reasons, diatoms need to be considered when discussing climate-change, ocean acidification and other global environmental phenomena.

Diatoms in biofilms

Just as diatoms, biofilms occur naturally in many different habitats with at least a small availability of water [Kolter & Greenberg, 2006]. Biofilms are aggregated microbial cells, which are often embedded in an EPS matrix and attached to a surface [Vert et al., 2012]. Many bacterial, archaeal and eukaryotic organisms besides the diatoms possess the capacity to form biofilms. The EPS matrix contains carbohydrates, proteins, proteoglycans, extracellular DNA, and modulates abiotic factors, such as moisture, electrochemistry, mechanical stability, and others [Flemming et al., 2007]. For example, it may adsorb nutrients from the surrounding medium, so that their availability within the biofilm is increased. Conversely, toxicants may be excluded, or sequestered to insoluble end stages [Hullebusch & Pechaud, 2015], so that their toxicity to cells within the biofilm is reduced. Because of these properties, biofilms offer favourable conditions for many species, including diatoms.

Biofilms are not only beneficial for the cells embedded in them, but also for outside organisms, as well as for the ecosystems as a whole. Primitive animals for example may have co-evolved with the O$_2$-producing cyanobacterial biofilms before the oxygenation of Earth’s oceans [Gingras et al., 2011]. More obvious and present is the utility of biofilms as a food source for grazing invertebrates [Poff & Ward, 1995], as well as fish [Carpentier et al., 2014] and even birds [Kuwae et al., 2008]. Humans utilise various types of biofilms not as food itself, but for food production, such as aquacultural shrimp rearing [Thompson et al., 2002] or abalone larvae settlement [Stott et al., 2004]. On the ecosystem level, phototrophic biofilms contribute to the mechanical stability of sediment due to their secretion of adhesive organic compounds (see Widdows & Brinsley [2002] for a review). Because biofilms fulfil such crucial ecosystem functions, their increased understanding, protection and appropriate application is a crucial part of environmental management by us humans.

Diatoms and other phototrophic organisms, of course depend on the availability of photosynthetically active radiation (PAR) in their habitat. Thus, diatom biofilms are most often found in riverine, lacustrine [Kwandrans, 2007] and intertidal [Sahan et al., 2007] ecosystems. Incidentally, these are the aquatic habitats that are also populated with anthropogenic structures. The undesired colonisation of man-made structures is called biofouling and negatively impacts shipping and other machinery. This impact occurs as increased hull drag, as higher maintenance costs due to clogging, as well as the costs of biofilm removal [Molino & Wetherbee, 2008]. Biofouling research has been conducted on the most prevalent biofouler species, and has achieved important insights into the biofilm formation processes. For example, the pre-conditioning of surfaces by heterotrophs speeds up the development of phototrophic biofilms [Roeselers et al., 2007], which in turn were found to
mediate the adherence of Bryozoa larvae [Dahms et al., 2004]. It is unclear how well the models of prokaryotic biofilm formation [Stoodley et al., 2002; Monds & O’Toole, 2009] can be applied to eukaryotes such as diatoms and other algae, but succession clearly occurs in underwater habitats just as it does in terrestrial ecosystems. There are anti-fouling strategies to inhibit the undesired formation of biofilms, but these are often accompanied by toxic side-effects on the environment [Karlsson et al., 2006]. Therefore, biofilm control strategies need to carefully balance economic interests with ecological consequences. Of particular interest are the chemical cues that foster the formation and resilience of biofilms, so that they may be countered in non-cytotoxic ways.

**Diatom-related signalling molecules and infochemicals**

In the soil, the surface of plant roots was recognised as the interface of complex biochemical interactions between plants and bacteria in the early 20th century, and summarily termed the “rhizosphere” [Hiltner, 1904; Hartmann et al., 2007]. There, both fungi and bacteria interact with the plant rhizome and each other, in both symbiotic and parasitic manners. In particular, the exchange of nutrients was understood to mutually influence the interaction partners and the microenvironment, in which they interact with each other. In the algae communities, this understanding was summarised by Bell & Mitchell [1972] in the “phycosphere” concept. Here too, the immediate surrounding of the cells is understood as the space of their interaction, which influences both their own co-evolution as well as their common impact on the ecosystem [Amin et al., 2012].

In particular, metabolites, toxins and other chemical cues mediate these interactions (see review by Ianora et al. [2006] and references therein). These chemical cues are often called “signalling” or “messenger” molecules, which implies evolution of the production and secretion mechanisms on the side of their producers, as well as sensing mechanisms on the side of the recipients. This surely is the case in many interactions, but in a broader ecological context, a more useful term may be “infochemical”. Infochemicals are understood from the point of effect on the recipient [Dicke & Sabelis, 1988], regardless of the specific nature or source of a chemical cue. The recipient has evolved to detect infochemicals, and to react to them. The information is in the olfactory pathways of the beholder, so to speak. Although infochemical production may not have evolved to fulfil an information transfer function, this possibility is of course not excluded from the understanding of chemical cues as infochemicals. For example, environmental phosphate availability has been linked to diatom-specific cyclin responses [Huysman et al., 2010]. Regardless of their source, phosphates can in this context be considered as infochemicals, to which diatoms can adjust the regulation of their cell cycle. In summary, infochemicals are naturally occurring substances that trigger a physiological or behavioural response in an organism to some aspect of its biotic or abiotic environment. However, non-natural chemicals can fulfil such functions as well (e.g. act as pheromones), and unintentionally influence the natural chemical communication. In ecology and ecotoxicology, this understanding is summarised under the term “infochemical effect” [Klaschka, 2008].

Both nutrients and toxins can be understood as infochemicals, because they both affect intracellular processes of the recipient. Examples for beneficial effects of infochemicals are the increased proliferation of certain bacteria when incubated together with diatoms [Grossart, 1999], and algae that depend on bacteria for essential vitamins [Croft et al., 2005]. Conversely, antibiotic infochemicals are for example algicidal chemicals produced by bacteria [Lovejoy et al., 1998], bacterial compounds that inhibit the swarming of other
bacteria [Böttcher & Clardy, 2014] and algal products that inhibit bacterial biofilm formation [Ren et al., 2002]. The types of the chemical mediators of these interactions are diverse: small molecules (above citations) as well as proteases have been found [Paul & Pohnert, 2011].

In the more specific context of diatoms, some chemical cues are released only upon cell death and then act as infochemicals to other organisms. For example, diatom cells that are damaged by grazing copepods release unsaturated aldehydes. These reduce the reproductive success of the copepods, and thus reduce the grazing load on the diatom culture in the long-term [Pohnert, 2005]. Such aldehydes have also been found to affect heat-shock protein expression in sea urchin embryos [Romano et al., 2011] and the transition from vegetative to reproductive stages in the development of sea squirt larva [Castellano et al., 2015]. The proposed mode of action of unsaturated aldehydes in such animals involves their intracellular nitric oxide (NO) messaging pathways to the effect that the aldehydes may reduce NO levels, which in turn may modify gene regulation downstream. Bruckner et al. [2011] reported that the secretion of extracellular organic chemicals by several freshwater diatoms is triggered by bacterial infochemicals. Their chemical identification however, is ongoing. Recently, Amin et al. [2015] showed that Pseudo-nitzschia multiseries cell division is promoted by the Sulfitobacter-derived indole-3-acetic acid (IAA) in marine settings. Thus, specific chemical relationships between bacterial sources and diatom recipients are a current topic with high interests in the identification of chemical cues.

*Achnanthidium minutissimum* as a model organism

The diatom *A. minutissimum* (Kützing) Czarnecki [1994] is a cosmopolitan species complex of early colonisers in freshwater habitats [Round & Bukhtiyarova, 1996; Johnson et al., 1997; Potapova & Hamilton, 2007]. *A. minutissimum* is found in the littoral zone along the shore of Lake Constance, where it forms photoautotrophic–heterotrophic biofilm communities with satellite bacteria [Bahulikar, 2006]. Such bacteria have previously been shown to modulate *A. minutissimum*’s organic secretions such as EPS and amino acids [Bruckner et al., 2008, 2011]. Because these modulations occurred also upon treatment with spent bacterial medium, *A. minutissimum* apparently reacts to soluble chemical substances produced by the bacteria. This diatom’s fast colonisation of new substrates is facilitated by the quick formation of adherence-providing EPS structures such as stalks. These structures consist of aggregated EPS at the apical part of a cell, which grow into a shaft [Wang et al., 1997]. Besides stalks, *A. minutissimum* also produces EPS capsules, which envelope the whole diatom cell (Fig. 1.1). In contrast to stalks, capsules appear later in the culture’s growth phase, but their function is less apparent and may be manifold [Lewin, 1955; Geitler, 1977]. Capsules have been suggested to play a role in substrate attachment, nutrient capture, reproduction, and grazer defence. Additionally, they appear to provide *A. minutissimum* with a mechanical barrier against bacteria [Windler et al., 2012].

*A. minutissimum* has been established as a laboratory model organism in the form of xenic cultures (i.e. associated with naturally co-occurring bacteria) and axenified suspension cultures [Myklestad et al., 1989; Windler et al., 2012]. Moreover, *A. minutissimum* has been used as an *in situ* biomonitor for heavy metals in the environment. Frustules react to increased heavy metal levels by deformations, which can be quantified microscopically [Morin et al., 2008; Falasco et al., 2009; Cantonati et al., 2014]. *A. minutissimum* can also be useful to archaeologists, because its abundance was found to correlate with
Correlative light-electron microscopy (CLEM)

Light microscopy (LM) and electron microscopy (EM) have been developed up to their respective physical limits largely independently. Utilising stimulated fluorescence emission and depletion, LM has even breached the previously dogmatised Abbe diffraction limit [Klar & Hell, 1999; Hell, 2009]. Correlation techniques between these two microscopy methods enable a two-step approach to many imaging projects: observe many events or structures of interest using LM, and subsequently investigate structural details by EM (see Mironov & Beznoussenko [2009] for a review). CLEM techniques have been applied to a wide variety of sample types: from crystal grains [Wilding & Geissinger, 1973] to small model animals [Kolotuev et al., 2010]. The correlation results from the ability to find the same locations within a sample with both microscope types. Several techniques are able to facilitate the correlation (reviewed by Sosinsky et al. [2007]), such as utilising landmarks within the sample or the direct labelling of the locations of interest. Using landmarks, one has to take into account that samples structures may deform due to the harsh EM preparation procedures of fixating, freezing and drying [Hoagland et al., 1993]. Direct labelling requires staining of the structures of interest with for example green fluorescent protein (GFP) and other fluorophores. These can double as catalysts for the photo-oxidation of 3',3'-diaminobenzidine into a polymeric precipitate, which can in turn be stained with the electron-dense osmium tetroxid [Maranto, 1982; Grabenbauer,
2012]. Because proteins can be tagged with GFP, many intra- and subcellular structures become observable by CLEM. In the present thesis, observations were focussed on complete diatom cells, and a minimally invasive correlation technique for their in situ marking in biofilms was tested.

**Research objectives**

This thesis approaches the chemical communication of diatoms and bacteria from a methodological standpoint. It is the opinion of the author that the quality of scientific work is determined largely by the quality of the available techniques and tools. It therefore became a central theme of this thesis to develop and improve methods of investigating biofilms, and to make them available to the research community.

Chapter 2 will present a biofilm model system based on A. minutissimum and a Bacteroidetes strain. The model system was developed to advance the screening of a variety of sample types for biofilm-inducing effects. We additionally asked, which influence the bacterium has on the EPS production of the diatom.

Chapter 3 will present work on the up-scaling and fractionation of bacterial supernatants, guided by the afore-mentioned bioassay. It was hypothesised that a multi-step liquid-liquid extraction combined with a solid-phase extraction could extract biofilm-inducing fractions from bacterial supernatant.

Chapter 4 will explain workflow automations and optimisations. How to remove bottlenecks in the measurement and data processing? And how to increase sample throughputs and replicate numbers? Our answers to these questions enhance the applicability of the bioassay for large sample sets, such as bacterial mutant strains.

Chapter 5 will take an electron microscopic view into the A. minutissimum biofilms, and elucidate the microstructure of this diatom’s EPS capsule. We solved the problem of finding the exact same cells in both light and electron microscopy with a simple, biofilm-compatible technique. Moreover, a novel microstructure type in freshwater diatoms will be described, and a model for the capsule formation will be proposed.
Chapter 2

Biofilm and capsule formation of the diatom *Achnanthidium minutissimum* are affected by a bacterium

Miriam Windler\(^1\), Katrin Leinweber\(^2\), Carolina Rio Bartulos\(^3\), Bodo Philipp\(^4\) & Peter G. Kroth\(^3\)

Abstract

Photoautotrophic biofilms play an important role in various aquatic habitats and are composed of prokaryotic and/or eukaryotic organisms embedded in extracellular polymeric substances (EPS). We have isolated diatoms as well as bacteria from freshwater biofilms to study organismal interactions between representative isolates. We found that bacteria have a strong impact on the biofilm formation of the pennate diatom *A. minutissimum*. This alga produces extracellular capsules of insoluble EPS, mostly carbohydrates (CHO), only in the presence of bacteria (xenic culture). The EPS themselves also have a strong impact on the aggregation and attachment of the algae. In the absence of bacteria (axenic culture), *A. minutissimum* did not form capsules and the cells grew completely suspended. Fractionation and quantification of CHO revealed that the diatom in axenic culture produces large amounts of soluble CHO, whereas in the xenic culture mainly insoluble CHO were detected. For investigation of biofilm formation by *A. minutissimum*, a bioassay was established using a diatom satellite Bacteroidetes bacterium that had been shown to induce capsule formation of *A. minutissimum*. Interestingly, capsule and biofilm induction can be achieved by addition of bacterial spent medium, indicating that soluble hydrophobic molecules produced by the bacterium may mediate the diatom/bacteria interaction. With the designed bioassay, a reliable tool is now available to study the chemical interactions between diatoms and bacteria with consequences for biofilm formation.

Keywords

*Achnanthidium*, biofilm, capsule, diatom, EPS

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CHAPTER 2. A. MINUTISSIMUM BIOFILM FORMATION

Introduction

Photoautotrophic biofilms are a typical feature in the littoral zones of lakes, streams and oceans. Stones or any other substrata can be covered by a brownish or greenish mucous layer, whenever sufficient light and water is available. These biofilms are a habitat of high primary production [Wetzel, 1964] and may be responsible for sediment stabilization [Cooksey & Wigglesworth-Cooksey, 1995]. However, biofilms also have negative effects, as for instance biofouling on human made surfaces like ship hulls or pipes causes high costs in shipping and water management [Gaylarde & Morton, 1999; Schultz et al., 2010; Wingender & Flemming, 2011]. In addition to the influence of exogenous factors like light, wave disturbance, temperature, water level fluctuations as well as grazing pressure [Hoagland & Peterson, 1990; Schmieder et al., 2004; Rao, 2010], the formation of photoautotrophic biofilms may be strongly influenced by the physiology of the inhabitants of the biofilms and their interactions [Bruckner et al., 2008]. Diatoms are common members and early colonisers of photoautotrophic biofilms [Cooksey & Wigglesworth-Cooksey, 1995; Wetherbee et al., 1998] and their productivity may have a strong influence on the whole biofilm. They can produce copious amounts of extracellular polymeric substances (EPS; Myklestad et al. [1989]) which are classified as cell bound EPS like stalks, tubes and capsules [Hoagland et al., 1993] or soluble EPS (SE). Diatoms are generally associated with bacteria belonging mostly to Alpha-, Beta- and Gamma-Proteobacteria, to the Cytophaga-Flavobacteria-Bacteroides (CFB) group and to Actinobacteria [Knoll et al., 2001; Sapp et al., 2007; Stanish et al., 2013]. Interactions between diatoms and bacteria may occur on different levels and may span from synergy via competition to parasitism or defence reactions [Amin et al., 2012]. Algae as primary producers provide organic substrates which serve as energy and carbon source for heterotrophic bacteria [Cole, 1982]. Bell & Mitchell [1972] introduced the “phycosphere” concept, describing the zone around the algal cell “in which bacterial growth is stimulated by extracellular products of the alga.” The diatoms may in turn require essential compounds from the bacteria (e.g. vitamins; Croft et al. [2005]). Little is known about the molecular processes underlying diatom/bacteria interactions within these biofilm communities. Biofilm inhabitants may affect the physiology of other organisms by soluble molecules, indicating that many of these inter-species interactions are based on chemical signals released by diatoms and by bacteria. Thomas & Robinson [1987] observed that the exudates of the xenic diatom *Amphora coffeaeformis* lead to enhanced tolerance of the diatom against copper and tributyltin fluoride. This suggests that either bacterial substances themselves or algal exudates induced by bacteria may trigger the stress response of *A. coffeaeformis*. Such unknown substances may act as signals that mediate recognition and communication between the interaction partners or directly cause a specific effect as toxic compounds. Amin et al. [2012] suggested that substances which are used for intra-species communication, like autoinducers in bacterial quorum sensing or pheromones in the case of diatoms, might also be involved in interactions between diatoms and bacteria. Such interkingdom signalling was already described to play a role for the seaweed *Ulva*, where zoospores are attracted by bacterial biofilms via released N-acyl-L-homoserine lactones (AHLs; Joint et al. [2007]). These AHLs are common autoinducers of Gram-negative bacteria [Chhabra et al., 2005]. However, as Amin et al. [2012] stated, a reliable bioassay comprising a diatom-bacterium pair with a stable interdependency is needed to elucidate the molecular and chemical basis of these interactions.

The goal of this study was to establish a model system for studying the interaction of benthic diatoms and bacteria during biofilm formation. A basic requirement for the inves-
tigation of a diatom/bacterium pair is the demonstration of a strong phenotype change in the diatom when cultivated in presence or absence of the bacterium. The model organisms utilised here, *A. minutissimum* (Kützing) Czarnecki and Bacteroidetes strain 32, have been isolated by us from photoautotrophic, epilithic biofilms taken from the littoral zone of Lake Constance. *A. minutissimum* (renamed from *Achnanthes minutissima* (Kützing) Czarnecki [1994]) is one of the most abundant freshwater diatoms [Patrick & Reimer, 1966; Krammer & Lange-Bertalot, 1991]. It is frequently found in epilithic biofilms of Lake Constance and represents a eukaryotic pioneer during the initial processes of biofilm formation [Sekar et al., 2004]. Common EPS structures of this diatom are stalks and capsules [Geitler, 1977] which were described in the literature as “unidirectionally deposited, multilayered structures attaching cells to substrata” for stalks and “material external to the wall and often sloughed into the environment” for capsules [Hoagland et al., 1993]. Bacteria of the Bacteroidetes phylum are frequently associated with diatoms [Amin et al., 2012] and were also found to be prominent in xenic diatom isolates from photoautotrophic epilithic biofilms of Lake Constance [Bruckner et al., 2008]. Bacteroidetes strain 32 belongs to the *Dyadobacter* genus. Bacteria of this group were found in freshwater, soil samples, or to be associated with maize [Chelius & Triplett, 2000; Baik et al., 2007; Zhang et al., 2010].

**Materials and methods**

**Organisms and cultivation conditions**

*Ankistrodesmus minutissimus* (Kützing) Czarnecki was isolated from photoautotrophic epilithic biofilms of Lake Constance [Windler et al., 2012]. The diatom was either cultivated with co-isolated bacteria as “xenic culture” or additionally as “axenic culture” after removal of associated bacteria as described earlier [Windler et al., 2012]. The diatom stock cultures were cultivated in a modified liquid Bacillariophycean Medium (BM; Windler et al. [2012]) in cell culture flasks with ventilation caps (Sarstedt, Newton, NC, USA), in which the cells could form a biofilm on the vessel surface. Monthly, these cultures were scraped off and sub-cultured in new BM. For aggregation, the xenic and axenic diatom cultures were cultivated at 100 rpm on an orbital shaker (type 3019; GFL, Burgwedel, Germany). The diatom cultures were exposed to a 12:12 h light:dark cycle with a light intensity of 20-50 \(\text{umol} \text{photons m}^{-2} \text{s}^{-1}\) at 16°C (in this study denoted as standard cultivation conditions for diatoms).

Bacteroidetes strain 32 was isolated by Bruckner et al. [2008]. This bacterium was enriched from a xenic *Cymbella microcephala* Grunow strain D-32 culture, which originated from photoautotrophic, epilithic biofilms from the same sampling site as *A. minutissimum* (47°41′ N; 9°11′ E, Germany). The bacterium was cultivated at 22°C on agar plates containing 50% (v/v) Luria-Broth medium (diluted LB; Miller [1972]), sub-cultivated monthly, and stored at 4-8°C.

In the following, *A. minutissimum* together with all co-isolated bacteria is termed a “xenic culture”, whereas the purified diatom in co-culture with Bacteroidetes strain 32 is termed a “co-culture”.
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Bioassay for biofilm formation

*A. minutissimum* was cultivated either in the presence of bacteria, the sterile supernatant of Bacteroidetes strain 32, or single fractions of the respective bacterial supernatant. The assays were conducted in well plates (#677180, Cellstar); Greiner Bio-One, Frickenhausen, Germany. Axenic diatom cells were washed with fresh BM and $10^5$ to $2.8 \cdot 10^6 \text{cells} \cdot \text{mL}^{-1}$ were used per well, however, in each single experiment the same cell density was used for test cultures and control cultures. Bacterial cells, supernatant of the bacterial culture or single fractions of the bacterial supernatant, which were prepared as described below, were added and the cultures were complemented with BM to a total volume of 500 µL. Cultivation was performed at standard cultivation conditions for diatoms for at least 11 d and was stopped by gently removing the culture supernatant. Staining with CV solution or alcian blue was performed as described below.

Staining procedures and microscopy

Carbohydrates (CHO) associated with cells were stained either with alcian blue or CV. For CV staining, we used a 1:100 dilution of the Gram-staining reagent described by Kaplan & Fine [2002]. Supernatants of the diatom cultures were gently removed, the dye was added to the cultures and incubated for 1 min. Excessive dye was removed and the cultures were rinsed with water to remove non-attached cells [Izano et al., 2007]. To quantify biofilm formation, CV was extracted from the cultures with 1 mL ethanol and the absorption of the dye was determined photometrically at 580 nm. The alcian blue dye solution was modified according to Staats et al. [1999], here alcian blue (1% (w/v) in 3% acetic acid (Roth, Karlsruhe, Germany) was added and the cultures were washed once with water.

Cells were observed with an upright light microscope (Olympus BX51; Olympus Europe, Hamburg, Germany), equipped with the filter set 41020 (Chroma Technology Corp., Rockingham, VT, USA) or by using an inverted light microscope (Axiovert 40 C; Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were taken with the Zeiss Axio-Cam MRm or MRc digital camera systems (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany).

Co-cultivation with bacteria

For the co-cultivation experiments, Bacteroidetes strain 32 was grown in diluted LB (50% (v/v)), washed three times with BM to remove the bacterial medium and the bacterial suspension was adjusted to an optical density at 600 nm (OD$_{600}$) of 0.1, of which 5 µL were applied to the bioassay. The xenic *A. minutissimum* cultures were inoculated with the same chl concentration as the axenic cultures and the co-cultures (for chl measurement see below).

Incubation with bacterial spent medium

For those experiments requiring the sterile supernatant of the bacterial culture, Bacteroidetes strain 32 was cultivated in liquid BM supplemented with 10 mM glucose (glcBM) at 20°C and 135 rpm. The supernatant of the bacterial culture was harvested when the culture reached an OD$_{600}$ of 0.2. The culture was centrifuged at 5,525 g
Materials and Methods

(Allegra™ 25R centrifuge with TS-5.1-500 rotor and swinging buckets, 10 or 50 mL tubes, depending on the volume of the supernatant, Beckman Coulter, Krefeld, Germany) and the supernatant was filtered using a 0.2 µm filter (Filtropur S; Sarstedt, Nümbrecht, Germany). For filtration of large volumes of bacterial supernatant (>10 mL), the bacterial supernatant was additionally prefiltered through 3.0 µm membrane filter (Merck Millipore, Darmstadt, Germany) and 0.2 µm filter (Whatman, Dassel, Germany). The sterility of the filtrate was randomly tested by plating an aliquot on agar plates containing diluted LB medium (50% (v/v)) and incubated as described for Bacteroidetes strain 32. Different volumes of the sterile bacterial supernatant and equivalent volumes of glcBM for negative control were applied to the bioassay.

For fractionation, 10 mL of the bacterial supernatant were separated via solid-phase extraction (SPE) using C18-SPE endcapped cartridges (530 mg; Macherey-Nagel, Düren, Germany) according to Von Elert & Pohnert [2000]. The bacterial supernatant and medium control were adjusted to pH 7 with citric acid or sodium hydroxide. Flow-through (Ft), wash fraction (Wf) and eluate were collected separately. Ft and Wf were evaporated to near dryness and diluted in 1 mL BM. The eluate was evaporated to dryness to remove methanol and resuspended in 2 mL BM. Aliquots of this fraction were incubated for 1 h at 30°C and 80°C, respectively, for further dissolution of solid material. The bacterial growth medium (glcBM) was treated the same way and was used as a negative control. The unseparated bacterial supernatant was used as positive control.

For further fractionation, 910 mL of the sterile bacterial supernatant were loaded on an endcapped C18-SPE column (10 g; Macherey-Nagel) and were eluted stepwise with methanol at increasing concentrations (20%, 40%, 60%, 80%, and 100% methanol in ultrapure water). Each fraction was eluted with 20 mL of the solvent, except the 100% methanol fraction. This fraction was eluted with 25 mL. After evaporation, the residues were resuspended in 1 mL of ultrapure water, sterile filtered, and diluted in BM. 250 µL of the diluted fractions were tested for bioactivity.

The sterile bacterial supernatant was harvested at different growth phases of Bacteroidetes strain 32, which was cultivated in 100 mL glcBM in triplicates. 1.2 mL were removed at each time point. A total of 100 µL were used to measure OD₆₀₀ of the bacterium and the supernatant of the remaining volume was sterile filtered as described above. Half of the volume was frozen for determination of the glucose concentration via HPLC as described in Jagmann et al. [2010]. The other half volume was stored at 4°C until it was tested for bioactivity (50% (v/v) of the bacterial supernatants).

Growth of A. minutissimum in xenic and axenic cultures

Growth of axenic and xenic A. minutissimum and of the diatom co-cultured with Bacteroidetes strain 32 was measured. The bioassay was performed as described above and the chl concentrations of suspended cells in the supernatants (non-adherent cells) and of cells embedded in the biofilm (adherent cells) were determined as described below.
Quantification of soluble and bound EPS

Triplicates of xenic as well as axenic cultures were incubated in 100 mL BM in cell culture flasks at standard diatom cultivation conditions. The axenic cultures were inoculated at a cell density of 10^3 cells/mL. The chl concentrations were determined as described below and the xenic cultures were inoculated at the same chl concentrations as the axenic cultures. Soluble and bound EPS of xenic and axenic *A. minutissimum* were stepwise extracted according to the protocol established for isolation of “SE”-, “warm water soluble EPS (WW)”-, “hot water soluble EPS (HW)”-, “hot bicarbonate soluble EPS (HB)”-, and “hot alkali soluble EPS (HA)”-fractions according to Bahulikar & Kroth [2008] with modifications: The cells were scraped from the bottom of the tissue culture flasks and the suspensions were centrifuged for 20 min at 5,525 g and 20°C (Allegra™ 25R centrifuge with TS-5.1-500 rotor and 50 mL swinging buckets, Beckman Coulter). The supernatants containing the soluble CHO were carefully separated by decanting from the cell pellets containing the bound CHO. The supernatants were concentrated to 5 mL using a rotatory evaporator. Polymers were precipitated overnight in five volumes of 96% ethanol at -20°C. The precipitates were centrifuged for 20 min and 4°C at 4,300 g without active deceleration (Megafuge 1.0R with swing-out rotor 2705; Heraeus Instruments, Hanau, Germany), the pellets were dried under nitrogen gas and subsequently dissolved in 1 mL ultrapure water. To increase solubility, the suspensions were acidified with 2-10 µL concentrated H_2SO_4 and heated to 60-80°C when required to dissolve the pellets. The ethanol fractions containing the oligo-and monomers were evaporated and the pellets were dried and processed as described for the polymers.

The bound CHO were extracted stepwise. First, the cell pellets were resuspended in 1 mL sterile-filtered tap water to keep the osmolarity in the physiological range and incubated for 1 h at 30°C and 300 rpm. Cells were centrifuged (5,000 g for 10 min at 20°C, 5417R microcentrifuge; Eppendorf, Hamburg, Germany) and the supernatants containing the “WW” fraction were separated from the pellets. The following centrifugation steps were conducted at 5,000 g for 7 min at 4°C (5417R microcentrifuge; Eppendorf). Pellets were defatted by incubation in 1 mL 90% ethanol at 600 rpm for 15 min and subsequently centrifuged [Wustman et al., 1997; Bahulikar & Kroth, 2008]. This procedure was repeated 5-7 times until the pellets were colourless or slightly green. Cells were further washed twice with distilled water, then incubated in 1 mL distilled water at 90°C for 1 h and centrifuged. The supernatants were used for determination of the “HW” and the cell pellets were incubated for 1 h in 1 mL 0.5 M NaHCO_3 and 0.1 M EDTA at 95°C [Chiovitti et al., 2003] to harvest the “HB”. The remaining portion of the bound EPS was extracted by resuspending the pellets in 1 mL 1 M NaOH and 0.2 M NaNH_4 at 95°C for 1 h and subsequent centrifugation of the cell fragments, resulting in “HA”. Mono-/oligo- and polysaccharides of the soluble CHO fraction as well as the total CHO content of the WW-, HW-, HB-, and HA- fractions were measured using the phenol-sulfuric acid assay [Dubois et al., 1956] with glucose as standard according to De Brouwer et al. [2002] and Bruckner et al. [2008] and were specified as µg CHO/µg chl. In a follow-up experiment, we compared a standard curve for glucose with those of other CHO that were reported to be prevalent in *A. minutissimum* [Bahulikar & Kroth, 2008]. Glucose had the steepest regression slope (see Suppl. Fig. 6.6, p. 86), indicating that other CHO might have been slightly overestimated. Glucuronic acid was tested as well and was found to have the lowest regression slope (see Suppl. Fig. 6.5, p. 85). It is therefore not included in this study.
Determination of chlorophyll (chl) concentrations

Chl was extracted by addition of a mixture of 5% methanol and 95% acetone to the diatom cell pellet. The chl of surface-adherent cells was extracted by flushing the biofilm with the extraction mixture for several times directly in the cultivation well. Chl concentrations were determined using the equation and extinction coefficients for diatoms, chrysomonads, and brown algae according to Jeffrey & Humphrey [1975], and specified as total chl concentration (a sum of chl a and c).

Results

Differences in biofilm formation of xenic and axenic *A. minutissimum*

*A. minutissimum* is a benthic diatom growing attached to surfaces. Interestingly, we observed that xenic and axenic *A. minutissimum* cells showed very different aggregation behaviour. When cultivated on a shaker, the xenic *A. minutissimum* cells formed macroscopically visible aggregates (Fig. 2.1A), while the axenic diatom culture grew completely suspended and no aggregate formation was visible (Fig. 2.1B). Under non-shaking conditions, the xenic diatom cells were attached to the surface of the cultivation vessel, forming a biofilm. Microscopic analyses in combination with alcian blue staining revealed that the cells in the early and late stationary phase were surrounded by large capsules of bound EPS (Fig. 2.1, C and E). The respective diatom cells were attached to each other via the capsules, partially resulting in macroscopically visible aggregates. Cells of the axenic culture did not show any capsules, remaining freely dispersed and forming, if at all, only small aggregates of a few cells (Fig. 2.1, D and F). In addition to the capsules, the xenic cultures showed diffuse EPS structures that were stainable by alcian blue (Fig. 2.1C). This diffuse, unstructured form of EPS could also be found in the axenic culture (Fig. 2.1D).

Figure 2.1: Xenic (A, C, E) and axenic (B, D, F) cultures of the diatom *A. minutissimum*. The xenic cultures formed macroscopic visible aggregates when cultivated on a shaker (A), while the axenic cells grew suspended (B). Alcian blue stained cells with diffuse EPS in the xenic (C) and axenic (D) cultures and early capsule formation in the xenic culture in the early stationary phase. In the late stationary phase, capsules of the xenic culture are fully developed (E), but missing in the axenic culture (F). Autofluorescence of the chl appears as bright red area in the cells. Scale bars denote 10 µm.
Induction of capsule- and biofilm formation in the axenic *A. minutissimum* culture by co-cultivation with Bacteroidetes strain 32

A bioassay was designed to investigate the bacterial influence on capsulation/biofilm formation of *A. minutissimum*. Biofilm formation was quantified according to Izano et al. [2007], by staining of bound CHO with CV. The dye stains the cells as well as the bound EPS and its intensity depends on the amount of attached cells and insoluble EPS and therefore is proportional to the biomass. After staining, biofilm formation is visible by eye and biofilm quantity can be determined by measurement of the alcohol-extracted CV.

![Figure 2.2: Bioassay for biofilm formation. Induction of capsule- and biofilm formation in the axenic *A. minutissimum* culture. (A) Alcian blue or CV stained cultures of the diatom cultivated in a 48 well plate. Biofilms of the diatom in co-culture or treated with the sterile bacterial supernatant show a stronger staining, comparable to the xenic culture. The control cultures (Axenic and GlcBM) show only faint staining (*N* = 2). (B) Absorption of CV extracted from the axenic culture, the co-culture with Bacteroidetes strain 32 and the xenic diatom culture. (Caps) marks time points when capsules appeared (*N* = 3; error bars indicate standard deviation (SD)). (C) Microscopic images: Cells of the xenic culture, co-culture with Bacteroidetes strain 32 and axenic *A. minutissimum* culture stained with alcian blue after 21 d of cultivation. Arrows mark stalks of the diatom cells. Cell with stalk is pictured in the middle picture with 2-fold magnification. Scale bars denote 20 µm.
We found that the biofilm formation of the xenic culture and of the co-culture with Bacteroidetes strain 32 is accompanied by much higher CV staining compared with the axenic diatom culture (Fig. 2.2A). The stable biofilm of the xenic cultures allowed rough washing steps because the cells adhered strongly to the plastic surface of the wells, resulting in high CV absorption already after 3 d of cultivation (Fig. 2.2B). At that time, the chl concentrations were still relatively low in the xenic culture (0.38 µg/mL; see below Fig. 2.5A). The co-culture also exhibited strong biofilm formation after 3 d and it reached the highest values of CV adsorption after 10 d (Fig. 2.2B). After 3 d of cultivation the diatom cells in the xenic culture and in the co-culture exhibited stalks that apparently mediate adherence in early stages of biofilm formation (Fig. 2.2C). The cells in the xenic culture started capsule formation after 10 d, those of the co-culture after 14 d of cultivation, when cells were in the stationary phase. In the axenic culture, there was no biofilm and capsule formation observable even after 24 d. Here, the cells did not adhere to the well surface and were removed during the wash steps of the staining procedure. In some cases, CV did not stain the capsules properly when the biofilm was fully developed. In these cases, the cultures were additionally stained with alcian blue for microscopic observation and visualization of capsules in the xenic culture and co-culture (Fig. 2.2C).

Capsule and biofilm induction by the sterile supernatant of Bacteroidetes strain 32

Bacteroidetes strain 32 was cultivated in BM supplemented with 10 mM glucose (glcBM) before harvesting the spent medium. This medium allowed bacterial growth without inhibiting diatom growth as we observed for diluted LB. Capsule and biofilm formation of *A. minutissimum* can be induced by incubation of the axenic culture with the filtered sterile supernatant of Bacteroidetes strain 32 (Fig. 2.3). The intensities of CV adsorption of *A. minutissimum* cultures showed a dose-dependent pattern when treated with different volumes of the sterile bacterial supernatant and reached a maximal absorption after addition of 25% and 50% (v/v) of the bacterial supernatant (Fig. 2.3A). Diatom capsules were found in cultures which had been supplemented with 12.5%, 25%, and 50% (v/v) of the bacterial supernatant. Neither the control cultures, which had

Figure 2.3 (following page): *Induction of capsule and biofilm formation of A. minutissimum by sterile bacterial supernatant of Bacteroidetes strain 32.* Biofilms were stained with CV and the absorption of the dye was determined. Cultures exhibiting capsules are marked with (Caps). (A) Incubation with 6%, 12.5%, 25%, 50%, and 94% (v/v) of the sterile supernatant (N = 3). The control assays were conducted either with addition of equivalent volumes of the bacterial medium (glcBM) or the axenic diatom culture. (B) *A. minutissimum* incubated with different fractions of the C18-solid phase separated bacterial supernatant: eluate (Elu), eluate preheated to 30°C and 80°C, respectively (Elu 30/80°C), flow through (Ft) and wash fraction (Wf). Graphs show cultures treated with fractions which had been diluted to the original concentration. Fractions of the bacterial medium glcBM and the unseparated bacterial supernatant were used for negative and positive control (N = 1 for the fractions, N = 3 for the control). (C) *A. minutissimum* incubated with different fractions of the C18-SPE separated bacterial supernatant, eluted with increasing methanol concentrations (N = 2). Fractions of the bacterial growth medium glcBM were used for negative controls (lower curve). Error bars indicate SD.
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**A.** BIOFILM FORMATION

![Graph A](image1.png)

**B.** BIOFILM FORMATION

![Graph B](image2.png)

**C.** BIOFILM FORMATION

![Graph C](image3.png)
been treated with the glcBM only, nor the axenic culture did induce biofilm or capsule formation. This indicates that soluble molecules released by the bacterium induce biofilm and capsules of *A. minutissimum*.

For enrichment of the biofilm-inducing substance, we further separated the bacterial supernatant by solid phase extraction (SPE) according to Von Elert & Pohnert [2000]. The fractions were diluted to the original concentration prior to the application (Fig. 2.3B). The eluted fraction (Elu) showed a similar induction of capsule/biofilm formation as the unseparated bacterial spent medium (positive control). Ft and Wf of the extracted bacterial spent medium described above did not induce a stronger biofilm formation than the fractions of the bacterial growth medium, indicating that the biological activity had been completely absorbed by the nonpolar C18-sorbent and was released after extraction with methanol. Incubation of the bioactive fraction at 30°C and 80°C as well as evaporation to dryness did not diminish the biological activity. As the methanol that was used as solvent for the fractionation had been completely evaporated, we can exclude induction of capsule and biofilm formation by the alcohol itself. Furthermore, the SPE was performed with bacterial growth medium only. Here, no capsule or biofilm formation was observed in any of the fractions.

Figure 2.4: Biofilm induction according to different growth phases of *Bacteroidetes* strain 32. (A) Optical density (OD) of the bacterium in glcBM (black line) and simultaneous decrease of the glucose concentration (grey line). (B) Intensity of the CV extracted from *A. minutissimum* cultures treated with sterile supernatants of *Bacteroidetes* strain 32, harvested at different growth phases of the bacterium (given in OD). (Caps) indicates capsule formation. The diatom culture was treated with glcBM for control (*N* = 3; error bars indicate SD).

Furthermore, the bioactivity was found in fractions which were eluted with high methanol concentrations, thus increased biofilm formation was mainly found in *A. minutissimum* cultures which had been treated with the 60%-100% methanol fractions and capsules were only found in the 80%-100% methanol fractions (Fig. 2.3C). These results suggest a nonpolar character of the bioactive molecule, as well as resistance to heat and dryness. The observed effect of bacterial supernatant on *A. minutissimum* is very reproducible, we were able to repeat the experiments several times independently starting from different bacterial cultures and purifying the bioactive compound(s) to varying degrees (data not shown).

To investigate the optimal growth phase of the bacterium for sufficient production of the bioactive substance, the bacterial supernatant was harvested at different time points and
each supernatant was tested for bioactivity. Figure 2.4A shows the growth of Bacteroidetes strain 32 in glcBM reaching an OD$_{600}$ of 0.24 in the stationary phase. Glucose consumption, as determined via HPLC analysis, resulted in a decrease of the glucose concentration from 10.3 to 8.5 mM during this time. Biofilm formation of A. minutissimum, as based on an increase in extractable CV adsorption from 0.16 to 0.35, was already induced by bacterial supernatant taken in the early exponential phase of the bacterium at an OD$_{600}$ of 0.016 (Fig. 2.4B). Accordingly, capsule formation was only induced by supernatants taken after the bacterium reached the mid-exponential phase at an OD$_{600}$ of 0.03.

Growth of A. minutissimum in xenic and axenic cultures

Growth of A. minutissimum in the bioassays was determined by measuring the chl concentrations (Fig. 2.5). This method was chosen, because both co-culture and xenic culture tended to form cell aggregates, which hampered reproducible cell counting. The xenic culture reached a similar maximum chl content as the xenic culture and the co-culture (0.78±0.13 µg/mL after 10 d for the xenic culture, 0.86±0.05 µg/mL and 0.81±0.06 µg/mL after 7 d for the xenic culture and co-culture, respectively; Fig. 2.5A). Interestingly, the chl content of the xenic culture and of the co-culture showed a plateau in the stationary phase after 17 d of cultivation, whereas the chl concentration of the xenic culture decreased continuously. In the xenic culture and in the co-culture, the main chl concentrations were found in the biofilm fraction, indicating that these cultures consist mostly of adherent cells (Fig. 2.5A, B and C). Nearly no chl was detected in the supernatant after 10 and 14 d of cultivation, respectively, at a time when the cells exhibit capsules. In contrast, most of the chl of the axenic culture was found in the non-adherent fraction (Fig. 2.5D). The bacterium itself was not able to grow without the diatom in the diatom full medium (BM; Suppl. Fig. 6.7, p. 87).

Analysis of carbohydrates

We analysed the CHO content and quality by stepwise extraction of soluble and insoluble EPS from xenic and axenic A. minutissimum contents as described in Materials and methods. Quantification of CHO in each fraction showed that in the late exponential growth phase (after 12 d of cultivation), the axenic and xenic cultures exhibited similar amounts of bound CHO (Fig. 2.6A). At that time, the xenic diatoms did not show capsules and the axenic cells secreted about 5-fold more soluble CHO compared with the xenic culture. In the early stationary phase, after 20 d of cultivation, the amount of bound CHO in the HB fraction of the xenic culture increased (Fig. 2.6B) and light microscopy indicated that the xenic cells started to form capsules (Fig. 2.1C). The capsules were fully developed in the late stationary phase, after 33 d, when the HW and HB fractions exhibited large amounts of insoluble CHO (Figs. 2.1E and 2.6C). Microscopic observations revealed that dissolution of capsular material correlated well with an increase in CHO content in the HW and HB fractions (Suppl. Fig. 6.8, p. 87). Thus, the capsular material of the xenic culture can be extracted with the hot water and hot bicarbonate treatments, leaving only very little CHO material in the hot alkali fraction. During the whole cultivation period, the amount of soluble CHO remained relatively low in the xenic culture, especially in the monomer fractions (Fig. 2.6). The axenic A. minutissimum, on the other hand, showed less CHO in the insoluble fractions but produced large amounts of soluble CHO.
Figure 2.5: Chl concentrations of *A. minutissimum* in axenic and xenic conditions and in the co-culture with Bacteroidetes strain 32. (A) Sum of the chl concentrations of adherent and non-adherent cells of the cultures. (B) chl concentrations of adherent and non-adherent cells of the xenic culture, (C) of the co-culture with Bacteroidetes strain 32, and (D) of the axenic culture. Capsules (Caps) in the xenic and co-culture are formed after 10 and 14 d of cultivation, respectively (*N* = 3; error bars indicate standard deviation).
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Discussion

When cultivated in presence or absence of distinct bacteria, the diatom A. minutissimum showed a considerably different behaviour regarding cell aggregation, cell attachment and biofilm formation, which is most likely because of changes in the generation of soluble and insoluble EPS by the diatom. Positive as well as negative bacterial influences on aggregation formation have been described in the context of marine snow, aggregated material of cells, and organic matter in the oceans [Grossart et al., 2006; Gärdes et al., 2011], and several possible mechanisms have been discussed. Bacteria may increase the amount of matrix material either by contribution of bacterial EPS [Decho, 1990] or by stimulation of EPS secretion by the algae. Also bacterial modification of organic material and subsequent changes of adhesive properties has been suggested [Grossart et al., 2005]. Cellular aggregation is of ecological relevance as it increases the sinking velocity and thus plays an important role in the flux of organic matter into deeper parts of the water column [Fowler & Knauer, 1986; Gärdes et al., 2011]. It also may alter the consumption by grazers and thereby the transfer of organic matter to upper trophic levels [Decho, 1990].

Xenic A. minutissimum cells may stick to the well surface because of development of capsules of extracellular organic matter, while axenic cultures revealed no such capsules and grew suspended. Capsule and biofilm formation of axenic A. minutissimum, however, can be induced by addition of Bacteroidetes strain 32. Of eight other tested bacterial isolates, several led to stronger biofilm formation, but none of them was able to induce capsule formation (Suppl. Fig. 6.9, p. 89). We made similar observations in earlier experiments showing that capsuleation and altered EPS structures of the freshwater biofilm diatom Cymbella microcephala occur in co-culture with Bacteroidetes strain 32, indicating that this might be a specific property of the bacterium [Bruckner et al., 2008].

A. minutissimum also shows capsule formation when treated with the sterile supernatant of Bacteroidetes strain 32. Apparently, the diatom is able to recognise one or more soluble molecules produced by the bacterium; a direct cell-to-cell contact between the interaction partners apparently is not required as it was shown previously for Pseudo-nitzschia multiseries [Kobayashi et al., 2009]. Interestingly, Bacteroidetes strain 32 produced the putative infochemicals even when cultivated separately, which indicates a constitutive production and secretion. As the biotic activity of this or these substances can be demonstrated already in the early exponential growth phase of Bacteroidetes strain 32, they apparently are produced by intact bacterial cells and do not constitute substances that are released after cell damage. As shown in figure 2.4B, A. minutissimum reacts with a sharp increase in biofilm formation even at relatively low bacterial abundance, thus either the diatom is very sensitive to the substance(s) or the production to the substance(s) is

Figure 2.6 (following page): Quantification of soluble and bound carbohydrates of axenic and xenic A. minutissimum cultures. Specific carbohydrate quantities are shown for axenic and xenic cultures and specified in µg CHO/µg chl. Carbohydrates were extracted in different growth phases of the diatom: late-exponential growth phase (12 d of cultivation), early stationary phase (20 d of cultivation), and late stationary phase (33 d of cultivation). Soluble carbohydrates were measured as soluble polymers and monomers. Bound carbohydrates were stepwise extracted with warm water (WW), hot water (HW), hot bicarbonate (HB), and hot alkali (HA; N = 3; error bars indicate SD).
DISCUSSION

Late exponential phase (12 d, no capsule formation)

Late stationary phase (33 d, capsules are fully developed)

Early stationary phase (20 d, start of capsule formation)
strongly increased once the bacterial density exceeds a certain threshold. The bioactive molecules were extractable via reversed phase cartridges indicating a hydrophobic character, similarly to AHLs, a major class of autoinducers produced by Gram-negative bacteria [Chhabra et al., 2005]. The inducibility of capsule formation using spent medium of the bacterium shows that the diatom itself is the active producer of biofilm material and the active contribution of bacterial EPS can therefore be excluded.

Former studies showed that surface materials may influence diatom attachment and biofilm formation [Gawne et al., 1998; Mieszkin et al., 2012]. Polystyrene for example, represents a hydrophobic surface and was found to be preferred out of four different materials for attachment of the diatom Achnanthes longipes [Gawne et al., 1998]. In our study, axenic A. minutissimum did not attach to polystyrene of the well plates and hence attachment only depends on Bacteroidetes strain 32 or its spent medium. Gawne et al. [1998] and Mieszkin et al. [2012] further showed that bacterial biofilms on surfaces may influence attachment of diatoms in a positive or negative way and often precedes biofilm formation of diatoms. As biofilm of A. minutissimum can be also induced by the sterile supernatant of the bacterium, preceding biofilm formation of the bacterium as a sort of base layer is obviously not the only reason for attachment of the alga.

CHO quantification revealed that the axenic diatom culture contained large amounts of soluble CHO. In the late stationary phase, we found variable amounts of soluble polymers in this culture (15.9, 11.1, and 55.6 µg CHO µg chl; Fig. 2.6C), resulting in a high standard deviation. From former experiments, we know that the axenic cultures usually secrete large amounts of soluble polymeric CHO in the stationary phase (up to 10⁵ µg CHO µg chl; Suppl. Fig. 6.8, p. 87). However, in the xenic culture insoluble CHO were dominant, especially in the late-stationary phase when the capsules were fully developed. In this growth phase, CHO of the xenic culture were primarily found in the HW and HB fractions, which correlates well with the dissolution of the capsular material (Suppl. Fig. 6.8 C and D, p. 87), implying that the CHO content of these fractions derives from capsular material. Absolute values of the CHO extraction have to be interpreted with caution, as the extraction of bound CHO with warm water is controversially discussed in the literature because of possible contamination with released intracellular CHO [Chiovitti et al., 2003; De Brouwer & Stal, 2004]. However, we found only little amounts of CHO in this fraction (<2 µg CHO µg chl) compared to the other fractions. We tried to minimise possible contamination with intracellular CHO previous to the extraction with warm water as we defatted the cells with ethanol, which was shown to remove intracellular material [Wustman et al., 1997]. We checked the reliability of the defatting step and found no CHO left after two extractions with ethanol, suggesting that all intracellular CHO were removed. Here, axenic and xenic cells released similar amounts of CHO during the defatting steps (see Suppl. Fig. 6.10, p. 90), while chl was efficiently removed (see Suppl. Fig. 6.11, p. 91).

Glucose has been used as standard for CHO measurement as described earlier [De Brouwer et al., 2002; Bruckner et al., 2008]. To evaluate the difference with the naturally occurring CHO mix of A. minutissimum, we tested six most abundant compounds which were found in A. minutissimum previously [Bahulikar & Kroth, 2008] using the Dubois method (Suppl. Figs. 6.6, p. 86 and 6.5, p. 85). These sugars yielded a somewhat weaker colorimetric reaction compared with glucose. Thus, the absolute values of the CHO fractions may be overestimated, however, our findings are based on a comparison of CHO contents in xenic and axenic cultures and not on absolute values.

As bacteria in the xenic culture potentially may consume the soluble CHO (especially the easily accessible monomers, whereas the bound CHO may be more resistant to bacterial
degradation), we cannot estimate whether the total amounts of CHO in xenic and axenic cultures are comparable. However, it might be possible that axenic and xenic cultures produce similar amounts of CHO, but the condition of the secreted CHO changed from a dissolved to an insoluble state in the presence of bacteria. Nonetheless, in both cases it is evident that *A. minutissimum* strongly secreted CHO even when no structured EPS was visible. When nutrients become limited–thus within the stationary phase–increased EPS production and capsule formation is a known phenomenon [Lewin, 1955; Bhosle et al., 1995] and is assumed to serve also as an overflow mechanism [Staats et al., 2000].

The exact function of capsules produced by *A. minutissimum* is still unclear. Geitler [1977] proposed that capsule formation of this alga is involved in sexual reproduction, whereas pads and stalks are regarded as common structures of vegetative cells. This proposal was based on the observation that the capsule may engulf both mating partners, however, we also found single cells to be surrounded by a capsule. According to Lewin [1955] the production of cell bound gelatinous matter may provide attachment, which is supported by our observation that cells exhibiting capsules were attached to each other or to artificial surfaces. The cells of *A. minutissimum* are motile until they form capsules. These capsules can be stained with alcian blue in acetic acid, which stains anionic polyesaccharides [Staats et al., 1999] that are thought to facilitate adhesion [Dade et al., 1990]. However, in the xenic culture and in the co-culture with Bacteroidetes strain 32, the diatom cells within a few days adhered to the surface mediated by stalks before a capsule became visible. We therefore conclude that bacterially induced capsulation may not be a prerequisite for surface-attachment, but possibly for strengthening the attachment and for irreversible binding. Diffuse EPS, also known as transparent exopolymer particles (Passow [2002] observed both in the axenic and xenic cultures, obviously did not facilitate surface adhesion, as axenic cells can be easily removed during the washing procedures. Surface adhesion of diatoms could be relevant once the cells found a favourable environment e.g., when they recognise the presence of an interaction partner. This would implicate a mutualistic character of the interaction with Bacteroidetes strain 32. Indeed, the bacterium was not able to grow in BM alone, suggesting that exudates from the diatom are utilised as carbon sources. The finding of a clearly higher abundance of bacteria in co-culture with diatoms compared with the bacterial growth in the respective diatom medium, supports that this is also the case for exudates from other diatom species [Grossart et al., 2006; Gärdes et al., 2011]. Thus, a further explanation for capsulation in the presence of bacteria could be that the capsule itself may serve as a feeding ground for the bacteria and may help to keep the bacteria in close proximity to the diatom cell. It is tempting to speculate that bacteria may induce cell aggregation and biofilm formation of the diatom to keep the alga in spatial proximity and to ensure an adequate nutrient supply by the primary producer. The diatom in turn may benefit from the general properties of the biofilm, e.g., protection from toxic compounds [Ceri et al., 1999], UV radiation [Ehling-Schulz et al., 1997], and grazer protection. They may also directly benefit from the bacterium, for example, by bacterial supply of essential nutrients (e.g., vitamins). The medium used in this study indeed contains sufficient basic nutrients for unlimited diatom growth. However, once a biofilm is established, local nutrient deficiency may occur which may be compensated by bacteria.

Microscopic analyses revealed that the bacterial cells of the xenic culture most likely cannot penetrate the capsule, which is visible by a bacteria-free area around the diatom cell (data not shown), and thus the diatom may prevent being overgrown or parasitised by bacteria. This may be much more evident when the diatom cells are stressed and thus much more vulnerable. In this context capsulation could also represent a defence mechanism.

DISCUSSION
The capsule could further protect the diatom cells from toxic bacterial compounds as EPS may reduce the susceptibility of biofilm organisms to some substances [Stewart & Costerton, 2001].

Conclusions

The bioassay for analysis of bacterial influence on biofilm structure and quantity of *A. minutissimum* established in this study proved to be very reliable. The enhanced biofilm formation is visible by eye after staining with CV, allowing a fast screening of a large number of substances as shown here for fractions of the separated bacterial supernatant. The assay could possibly be extended to test the impact of other chemicals on the biofilm formation of this or even other diatom species. *A. minutissimum* turned out to be an excellent model organism for the investigation of biofilm formation as it produces large amounts of soluble and bound EPS and it can be cryopreserved by using a modified protocol according to Buhmann et al. [2013]. The bioassay is based on the interaction of the alga with Bacteroidetes strain 32 as this bacterium induces the same physiological changes of capsulation and biofilm formation as shown for the satellite community of *A. minutissimum*. Up to now, little is known about the chemical interactions between diatoms and bacteria. The identification of the bioactive compound(s) of Bacteroidetes strain 32 and further physiological studies will be important to show whether the interaction between *A. minutissimum* and Bacteroidetes strain 32 has a mutualistic or an antibiotic character.

Acknowledgements

The authors gratefully acknowledge the financial support by the Konstanz Research School Chemical Biology (KoRS-CB, GSC 218) and the University of Konstanz. We also would like to thank Georg Pohnert (University of Jena) for very helpful discussions and Annette Ramsperger, Vera Bleicher, and Joana Thiel for technical support.
Chapter 3

A bioassay-guided fractionation of bacterial infochemicals that induce biofilm formation by Achnanthidium minutissimum

Katrin Leinweber¹, Dieter Spiteller¹ & Peter G. Kroth¹

Abstract

Few diatom infochemicals of bacterial origin have been purified and identified to date, and none in the biofilm model system comprising the Gram-negative Bacteroidetes strain 32 and the freshwater diatom A. minutissimum. After establishing this diatom in a laboratory biofilm model system, we tested a bioassay-guided fractionation concept based on liquid-liquid extraction and solid phase extraction of the sterilised, biofilm-inducing bacterial supernatant. The production of that supernatant and strain 32’s growth conditions were optimised based on the taxonomic placement of the Bacteroidetes strain. Liquid-liquid extraction was validated as a pre-purification step for the solid phase extraction, and was found to shift the fractions of interest towards the maximum of organic solvent, highlighting the need for further fractionation improvements. While the iterative improvement of the fractionation procedure was hindered by the assay’s long incubation time and variable responses to treatments with extract fractions, other application options emerged. This study may support the upscaling of bacteria infochemical discovery in the A. minutissimum biofilm model system.

¹ Department of Biology, University of Konstanz
CHAPTER 3. ASSAY-GUIDED INFOCHEMICAL FRACTIONATION

Introduction

Biofilms are a common mode of life for diatoms and bacteria in the phototrophic, littoral zone of Lake Constance [Bahulikar, 2006]. In order to study the interactions between diatoms and bacteria in biofilms, several model systems have been developed: First, Buhmann et al. [2012] built an incubator chamber with continuous medium flow and turbidity measurement, so that the spatial expansion of biofilms could be monitored and microscopically evaluated. Then, Windler et al. [2015] established a bioassay for the induction of *Achnanthes minutissima* biofilms by Bacteroidetes strain 32 and its spent culture supernatant. Both bioassays support the growth of xenic and axenic diatoms, as well as co-cultures of the latter with certain bacteria strains.

Bacteroidetes strain 32 (S32) was isolated from *Cymbella microcephala* biofilms by Bruckner et al. [2008], and shown to modulate the production of polysaccharides and dissolved free amino acids in diatoms [Bruckner et al., 2011]. Initial experiments with S32 and the biofilm-inducing activity of its supernatant found a constitutive secretion of the bioactive compounds throughout its growth cycle (see Fig. 2.4, p. 23). Initial fractionation steps suggested a hydrophobic nature (see Fig. 2.3, p. 21). Moreover, it is apparently not a protein, as it is resistant to heating to 60-80°C, freezing, drying and elution in highly concentrated methanol [Windler et al., 2014]. Denaturation and loss of bioactivity would be expected for most proteins. However, protein-based interactions between bacteria and diatoms exist as well [Paul & Pohnert, 2011]. Because of the above observations, and the apparent solubility of S32’s biofilm-inducing compound in the aqueous Bacillariophycean medium (BM), we hypothesised that the bioactivity is mediated by a small, hydrophobic compound, which also contain hydrophilic moieties (or a group of such molecules).

Pohnert et al. [2007] stated that approaches to the bioassay-guided identification of diatom-relevant signalling compounds and infochemicals is time consuming and requires reliable and unambiguous bioassay results. Guided by bioassays that fulfilled this requirement, pheromones in brown algae [Pohnert & Boland, 2002] and diatoms [Gillard et al., 2013], as well as diatom-derived zootoxins [Pohnert, 2005], have been identified. These studies have established that aquatic organisms are intricately linked by a complex network of chemically mediated interactions. One of the only bacterial molecules with an effect on diatoms that has also been structurally identified in natura is indole-3-acetic acid [Amin et al., 2015].

In order to prepare the identification of the biofilm-inducing compound(s) in our diatom-bacteria model system, we first scaled the secretion of S32’s biofilm-inducing compounds up. For this purpose, optimal growth conditions and media for maximum S32 cell densities were sought on the basis of the strain’s taxonomic placement. Moreover, we built on initial fractionation steps via solid-phase extraction [Windler et al., 2015], and compared the purity of several liquid-liquid extract fractions of the bacterial supernatant. Because these previous findings show that Bacteroidetes strain 32 secretes the bioactive substances constitutively even without *A. minutissimum*, we do not assume that they serve an active signalling role. Rather, they are considered here as “infochemicals” [Dicke & Sabelis, 1988], which the diatom senses and reacts to.
Materials and methods

Bacteroidetes S32 cultivation conditions and supernatant harvest

Liquid nitrogen stocks of Bacteroidetes strain 32 were re-established on agar plates prepared with 50% LB (lysogeny broth; Miller [1972]). S32 was allowed to grow for 5 days in darkness at 22°C. Afterwards, the plates were stored in darkness at 8°C. Transfer of S32 to new LB-agar plates commenced every 6 weeks. Liquid cultures of S32 for the supernatant harvests were grown in either Bacillariophycean medium (BM) supplemented with 10 mM glucose (gICBM) or in LB at a temperature of 20°C and shaking at 120 rpm for 5 to 7 days without particular darkening methods. Supernatant was obtained from the S32 liquid cultures by centrifugation at $5 \times 10^3 \, g$ for 5-10 min (Sorvall RC6 Plus, Thermo Scientific).

Sequencing of the 16S ribosomal RNA gene of Bacteroidetes strain 32

The 16S ribosomal RNA gene was amplified from a Bacteroidetes strain 32 colony standard primers “27f” and “1492r” (sequences in 3.1; ordered from Thermo Scientific). A “T Gradient” thermocycler (Biometra, Germany) conducted the PCRs as listed in Table 3.2. PCR products were run over gels of 1% agarose in 30 mL TAE buffer and supplemented with 1.5 μL RotiSafe GelStain (Carl Roth, Germany) for 10 min at 60 V, followed by 1 h at 90 V. Gel edges were cut off and visualised under UV light. Bands of interest (ca. 1.5 kb) were cut from the edge portions, so that these “rulers” could be used to excise the main band portions without direct UV exposure. DNA was purified from the gel pieces with the GeneClean Turbo kit (MP Biomedicals, Santa Ana, California, USA). Sequencing and automatic quality classification using PhredPhrap was performed by GATC (Konstanz, Germany). Sequences were manually assembled into a consensus, based on previous 16S sequencing runs of S32 in our lab. The 16S consensus sequence of S32 was searched against NCBI’s database of “16S ribosomal RNA sequences (Bacteria and Archaea)” with BLASTN 2.2.29+ [Zhang et al., 2000]. Additionally, the longest continuous sequence parts of good quality were analysed using the Ribosomal Database Project (RDP) Naïve Bayesian rRNA Classifier v2.10 [Wang et al., 2007] against the “16S rRNA training set 14” with a confidence threshold of 95%.

Table 3.1: Primers for amplification of bacterial 16S ribosomal RNA gene. Y denotes degenerate pyrimidine position.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27f</td>
<td>AGAGTTTGATCCTGGGCTCAG</td>
</tr>
<tr>
<td>1492r</td>
<td>TACGGYTACCTTACGACTT</td>
</tr>
</tbody>
</table>
CHAPTER 3. ASSAY-GUIDED INFOCHEMICAL FRACTIONATION

Table 3.2: **PCR cycling conditions for 16S rDNA amplification.** The sequence of denaturing, annealing and extending was conducted for 30 cycles.

<table>
<thead>
<tr>
<th>Step</th>
<th>T [°C]</th>
<th>t [min:sec]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>3:00</td>
</tr>
<tr>
<td>Denaturing</td>
<td>95</td>
<td>0:30</td>
</tr>
<tr>
<td>Annealing</td>
<td>53</td>
<td>0:30</td>
</tr>
<tr>
<td>Extending</td>
<td>72</td>
<td>1:40</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>5:00</td>
</tr>
</tbody>
</table>

Optimising Bacteroidetes strain 32 growth conditions

Cultivation conditions of *Dyadobacter* strains identified as closest known relatives of Bacteroidetes S32 were obtained from the catalogue of microorganisms of the Leibniz-Institut DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Media 1, 830, LB and B were prepared according to the recipes listed in tables 3.3 to 3.5, p. 34 to 35. Modified Bacillariophycean Medium (BM) was prepared as reported by Schlösser [1994] and Windler et al. [2012]. Glucose solution were added through a sterile 0.2 µm filter (Sarstedt, Germany) to a final concentration of 10 mM, yielding glcBM.

Liquid cultures in various media were inoculated with S32 cells that had been scraped off an agar plate and suspended in sterile-filtered tap water. Equal volumes of this S32 suspension were used to inoculate 10 mL medium in three to six sterile glass test tubes per temperature-medium combination. For each combination, one negative control with sterile medium was prepared as well. Directly after inoculation (day 0), an OD$_{600}$ measurement was taken in a CamSpec M107 spectrophotometer (Spectronic Camspec Ltd., Leeds, UK). The light path with the lowest OD$_{600}$ was determined for each test tube, marked on the glass, and subsequent measurements were conducted along it.

Table 3.3: **Recipe for 150 mL medium 1 (modified from DSMZ “nutrient agar”), adjusted to pH 7.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Manufacturer</th>
<th>Mass [g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>Roth, Germany</td>
<td>0.45</td>
</tr>
<tr>
<td>Meat extract</td>
<td>Merck, Germany</td>
<td>0.75</td>
</tr>
</tbody>
</table>
Table 3.4: Recipe for 150 mL medium 830 (modified from DSMZ “R2A”), adjusted to pH 7.2.

<table>
<thead>
<tr>
<th>Component</th>
<th>Manufacturer</th>
<th>Mass [mg]</th>
<th>Volume [µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvic acid</td>
<td>Sigma-Aldrich, Germany</td>
<td>-</td>
<td>28.42</td>
</tr>
<tr>
<td>K$_2$HPO$_4$ · 3 H$_2$O</td>
<td>Merck, Germany</td>
<td>59</td>
<td>-</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>Beckton Dickinson, USA</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>Merck, Germany</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>Merck, Germany</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Roth, Germany</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>LabScientific, USA</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>MgSO$_4$ · 7 H$_2$O</td>
<td>Merck, Germany</td>
<td>7.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.5: Recipe for 100 mL of the minimal medium B [Jagmann et al., 2010], modified with glucose instead of tryptone and yeast extract. Solid components were dissolved in MilliQ-H$_2$O, autoclaved and subsequently supplemented with the liquid components (see Table 3.6) through 0.2 µm filters.

<table>
<thead>
<tr>
<th>Component</th>
<th>Manufacturer</th>
<th>Mass [mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>Roth, Germany</td>
<td>1192</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>Fluka, Germany</td>
<td>27</td>
</tr>
<tr>
<td>MgSO$_4$ · 7 H$_2$O</td>
<td>Merck, Germany</td>
<td>24.6</td>
</tr>
<tr>
<td>KCl</td>
<td>Merck, Germany</td>
<td>104</td>
</tr>
<tr>
<td>NaCl</td>
<td>VWR, Europe</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 3.6: Liquid supplements for 100 mL minimal medium B (Table 3.5).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SL10$^2$</td>
<td>1000-fold</td>
<td>100</td>
<td>1-fold</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.1 M</td>
<td>10</td>
<td>0.01 mM</td>
</tr>
<tr>
<td>KNaPP$^3$</td>
<td>0.5 M</td>
<td>30</td>
<td>0.15 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.5 M</td>
<td>2</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

Table 3.7: Recipe for 150 mL LB.

<table>
<thead>
<tr>
<th>Component</th>
<th>Manufacturer</th>
<th>Mass [g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>VWR, Europe</td>
<td>1.5</td>
</tr>
<tr>
<td>Peptone</td>
<td>Roth, Germany</td>
<td>1.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Roth, Germany</td>
<td>0.75</td>
</tr>
</tbody>
</table>

$^2$ Trace element solution according to Widdel et al. [1983].

$^3$ Potassium sodium phosphate buffer composed of 0.35 mM K$_2$HPO$_4$ and 0.15 mM NaH$_2$PO$_4$ at pH 7-7.1.
CHAPTER 3. ASSAY-GUIDED INFOCHEMICAL FRACTIONATION

Liquid-liquid extraction of bacterial supernatants

Cell-free bacterial supernatants were adjusted to the pHs outlined in figure 3.2, p. 39 with HCl and NaOH and shaken against ethyl acetate (NORMAPUR, VWR) in appropriately large separating funnels for several minutes. In case phase separation was visibly hindered, either a few grams of NaCl were added and brought to dissolution by shaking again and/or the phase boundary and the ethyl acetate phase were centrifuged for 5 min at 5,000 g and separated by decanting. Final phases were either lyophilised at 0.1 mbar and 40°C (Alpha 2-4 LD plus with AVC 2-33 IR cold trap at -85°C; Martin Christ Gefriertrocknungsanlagen GmbH, Germany) or rotary-evaporated at 60°C (aqueous phases) or 40°C (ethyl acetate phases, after clearing of water with Na₂SO₄). Devices used for rotary evaporation were the Rotvapor R-210, Heating Bath B-491, Vacuum Pump V-710 and Vacuum Controller V-850. Residual solvents were removed at minimal pressures of the rotary evaporator or by membrane pump with a liquid nitrogen condensation trap. Dried extracts were weighed in round-bottomed flasks of known weights on Mettler AT261 DeltaRange balance (Mettler-Toledo GmbH, Gießen, Germany). Extracts of Bacteroidetes strain 32 supernatant obtained from glcBM culture supernatant were taken up in water and sterile filtered, while extracts from LB culture supernatant were taken up in methanol (MeOH).

C18-fractionation of liquid-liquid extracts

Solid phase extraction cartridges with octadecyl-(C18)-modified, endcapped silica (“Chromafix 10 g” and “Chromabond 530 mg”; Macherey-Nagel, Germany) were used according to the protocol established earlier in our lab (see ch. 2, p. 16). In case the dried liquid-liquid extracts were resolved in MeOH, they were not applied to the cartridges directly, but diluted to 10% (v/v) with MilliQ above the column material, so that a flow-through fraction could be collected. Washing solutions and eluents of different MeOH concentrations in MilliQ water were pre-mixed and applied to the cartridge manually. The pressure required to produce continuous droplet formation (a few per second) at the cartridge nozzle was applied manually or by compressed air. Care was taken to not let the cartridge material fall dry during fraction collection. Fractions were collected in glass tubes or round-bottomed flasks and dried by lyophilisation (fractions containing mostly MilliQ) or rotary evaporation (MeOH).

A. minutissimum bioassays

Bioassays for biofilm formation were conducted as described in chapter 2 (Bioassay for biofilm formation, p. 16) with the modification to always inoculate with 10⁵ diatom cells. Concentrated supernatant extracts were administered to approximately the same final concentration of 25-50% that was found to be optimal as shown in figure 2.3A, p. 21. However, reductions due to loss of substance during the extraction procedures can not be excluded. Untreated wells with only axenic A. minutissimum and/or those treated with extracts generated from blank medium by the same protocol as the samples served as negative controls.
RESULTS

Results

Optimisation of Bacteroidetes strain 32 growth conditions

The first goal in this study was the verification of the taxonomic placement of Bacteroidetes S32, in order to search for genus-specific, optimal cultivation conditions. These were envisioned to maximise the cell density of S32 in liquid cultures, which in turn should yield higher concentrations of the secreted biofilm inducer in the culture supernatant. The use of supernatant was continued according to Windler [2014] in order to avoid contamination of the extraction procedure with cellular material.

Figure 3.1: Growth of Bacteroidetes strain 32 in a selection of minimal (B & glcBM) and complex (LB, M1 & M830) media. See tables 3.3 to 3.7, pp. 34-35 for medium composition, and Windler et al. [2012] for the composition of modified liquid Bacillariophycean medium (BM), which was here supplemented with 10 mM glucose (glcBM). All data points represent average optical densities at 600 nm (OD<sub>600</sub>) normalised to the initial OD<sub>600</sub> of each culture directly after inoculation (day 0). Error bars represent standard deviations of 2σ across 3-6 replicates. No negative, sterile medium control displayed an increase in OD<sub>600</sub> at any temperature.

The 16S sequence obtained from Bacteroidetes S32 was classified as most closely related to the genus Dyadobacter (see Suppl., p. 92). Using BLAST, S32 clustered uniformly with several Dyadobacter species (see Suppl. Table 6.1, p. 94). Based on this taxonomic placement, the complex Dyadobacter media 1 (M1), 830 (M830) were tested together with LB for growth of Bacteroidetes strain 32 against the minimal media glcBM and B. As figure 3.1 shows, S32 cultures grew densest in LB at 20 and 30°C, with ca. 10-fold increases of OD<sub>600</sub> observed after 5-7 days. M830 yielded the next densest growth of strain 32. M1 and glcBM supported S32 growth to approximately the same, but low level. Medium B could only be tested at 30°C, but unlike the other media, it did not yield notable S32 cell densities. Bacteroidetes strain 32 reached the stationary phase fastest
in the glucose-supplemented Bacillariophycean medium (glcBM) and M830, with OD_{600} maxima reached after 3-5 days. In LB and M1, S32 required ca. 2 days longer to reach the stationary phase. In summary, media 1, 830 and B either did not yield higher S32 cell densities, or were more complex to prepare (see tables 3.3 to 3.7, pp. 34-35) than glcBM or LB. LB and the DSMZ-recommended temperatures of 20-30°C were chosen for further strain 32 cultivations. Growth in glcBM was in accordance with results by Windler [2014], so that this minimal medium should be considered for experiments which require quick but not dense Bacteroidetes strain 32 growth. Moreover, these were fail-safe and readily available cultivation conditions, thus supporting the goal to reproducibly up-scale the production of spent S32 culture supernatant with biofilm-inducing activity.

Liquid-liquid extraction of Bacteroidetes strain 32 supernatant

In parallel to the cell density optimisation, a liquid-liquid extraction of the bacterial supernatant from the previously used glcBM medium was designed and tested (Fig. 3.2). After the initial acidification of the supernatant to pH 2, liquid-liquid extraction commenced in several steps at alternating pH ranges. It was expected that the bioactivity would be transferred from acidic aqueous to ethyl acetate phases, and vice versa at the opposite pH range. The resulting aqueous and ethyl acetate phases (abbreviated as “AP” and “EAP”, followed by the pH value) were dried, taken up in BM or MeOH and tested in the _A. minutissimum_ bioassay for biofilm induction.

Two of these three liquid-liquid extraction steps were compared to the previously used, low-yield glcBM and the high-yield LB media (Fig. 3.3). As expected, the highest biofilm induction occurred in the co-cultures of axenic _A. minutissimum_ and Bacteroidetes strain 32 (S32). The lowest biofilm induction was observed for the aqueous phases of the first extraction of the acidified supernatant (AP2 glcBM) of a glcBM culture. The aqueous phase of the second extraction step (AP2-10 glcBM) induced a slightly higher biofilm signal. Samples that were alkalised first displayed lower biofilm induction (data not shown), suggesting that the bioactive compound did not possess basic moieties. Supernatant extracts from LB cultures produced the strongest biofilm formation, reaching similar levels as the co-culture.

The switch from glcBM to LB had to overcome the previously discovered limitation, that LB had growth-impeding effects on _A. minutissimum_ (see ch. 2, p. 21). Instead of resuspending dried residues from glcBM cultures in BM, MeOH was used to resuspend dried residues from LB cultures, because it had already proven to elute the biofilm- and capsule inducing activity from C18-columns in previous solid phase extraction experiments (see Fig. 2.3, p. 21). The resulting MeOH-dissolved samples improved the workflow of sample preparation and assay inoculation, because they could reasonably be assumed to be and remain sterile, thus making the sterile-filtration step redundant. Intriguingly, such extracts from LB that were not inoculated (negative bioassay controls) decreased the attachment of axenic _A. minutissimum_. This resulted in higher relative crystal violet intensity of biofilms triggered by extracts from LB supernatant, compared to supernatant from glcBM (Fig. 3.3). This might result from LB itself containing inhibiting substances, but whose effect was overcome by the biofilm inducers of Bacteroidetes strain 32.

The replacement of Bacillariophycean medium with methanol as the solvent for dried extract residues necessitated checking the effect of MeOH on the growth of the axenic, negative and xenic, positive _A. minutissimum_ control cultures (Fig. 3.4). Initially, the axenic cultures responded with a slight increase in chlorophyll content in the supernatant.
Figure 3.2: Liquid-liquid extraction workflow of Bacteroidetes strain 32 supernatant with fractionation of biofilm-inducing activity (grey). Rectangles represent samples of aqueous or ethyl acetate phase (AP and EAP, respectively). Hexagons represent work steps.
CHAPTER 3. ASSAY-GUIDED INFOCHEMICAL FRACTIONATION

Figure 3.3: Biofilm induction in axenic *A. minutissimum* cultures by aqueous phases of liquid-liquid extractions of Bacteroidetes strain 32 supernatant. **Left:** Quantification of crystal violet. (S32) Co-culture of axenic *A. minutissimum* and Bacteroidetes S32. (AP2) Acidic aqueous phase of first liquid-liquid extraction. (AP2-10 glcBM & LB) Basic aqueous phases of second extraction of S32 supernatant obtained from cultures grown in glucose-supplemented Bacillariophycean medium (glcBM) and LB, respectively. Error bars represent standard deviation of 2σ propagated from biological duplicates, each in technical triplicates. **Right:** Light microscopic image of a dense biofilm induced by the basic aqueous phase of the second extraction of S32 supernatant obtained from LB culture (AP2-10 LB). Scale bar represents 10 µm.

This meant that either more non-adherent cells were present in the cultures, or that those cells contained more chlorophyll. A search in the DiatomCyc.org database (as of July 23rd, 2015) revealed neither candidate pathways, nor enzymes for the possible utilisation of MeOH in the diatom *Phaeodactylum tricornutum* [Fabris et al., 2012]. Therefore, and because no such database of metabolic pathway exist for *A. minutissimum*, the cause of this unexpected initial result remains unclear. Larger control experiments with randomly placed replicates did not reproduce this chlorophyll increase, but generally showed a wide spread of chlorophyll concentrations (see Fig. 3.4a). Co-cultures of the diatom with Bacteroidetes strain 32 were tested in the same manner and showed no reduction of biofilm intensity in this MeOH concentration range.

Reassuringly, MeOH did not induce biofilms in the negative, axenic controls, nor did it disrupt biofilm formation in the positive, xenic controls at concentrations of up to 2-3% (v/v). Their biofilm intensities remained consistently on a higher level than those of the axenic cultures (Fig. 3.4b). Thus, the latter may have experienced some growth-inhibition by pure MeOH, but formed biofilms when treated with MeOH-dissolved supernatant extracts. We conclude that it is advisable to keep the MeOH load as low as reasonably pipettable in order to retain viable control cultures. However, it is relatively sure that in the low range of <3% MeOH in BM, the organic solvent does not disrupt the expected biofilm formation patterns.
In summary, the results of S32 growth experiments in various media (Fig. 3.1, p. 37), and the initial liquid-liquid extraction results of both glcBM- and LB-based extracts support (1) the concept of maximising supernatant production to obtain bioactive compounds and (2) the hypothesis, that the biofilm-inducing activity can be extracted with an organic solvent from acidified S32 supernatant. Due to the first point, the transition from low-yield glcBM to LB medium can be considered successful, while the second indicates that the bioactive molecule(s) may possess an acidic moiety.

Solid phase extraction of up-scaled liquid-liquid extracts of Bacteroidetes strain 32 supernatant

Next, the hydrophobic solid phase extraction (see ch. 2, p. 21) was repeated with the liquid-liquid extracted fractions of Bacteroidetes strain 32 supernatant from several-litre batches of liquid LB cultures grown to optical densities at 600 nm of 1-1.2. The initial results confirmed that the biofilm-inducing activity could be eluted from C18 with 60-100% MeOH (data not shown; similar to Fig. 2.3C, p. 21), but the varying purities of the different liquid-liquid extraction fractions were visible. The first ethyl acetate extract
from the supernatant at pH 2 for example (EAP2) dried to a more voluminous residue than the residues from the second (EAP2-10) and third extraction (EAP2-10-2).

The absorbance data from the extract samples was thus also normalised to the dry weights of the residues (Fig. 3.5). This highlights fractions with little residue, but strong biofilm induction. Seen in that light, the generally highest bioactivity with the least impurities was found in the 100% MeOH elution fractions. Of those, the third ethyl acetate phase (EAP2-10-2) contained the highest abundance of biofilm-inducing substance(s) relative to impurities in the residue. Unsurprisingly, the other fractions are ordered by the number of liquid-liquid extraction steps they underwent. The three-step liquid-liquid extraction may be considered successful, because the final extraction with ethyl acetate yielded an easier evaporation workflow, higher purity of the extracted sample and strong biofilm induction (as opposed to the previously used second aqueous phase).

![Figure 3.5: Biofilm induction of liquid-liquid extract fractions of Bacteroidetes strain 32 supernatant after extraction from C18 solid phase. All absorbance data points are normalised to the absorbance of the axenic control and to the dry weight of a fraction’s residue. Dashed, dark grey line (EAP2) represents C18-fractions of the first liquid-liquid extract of supernatant (ethyl acetate phase from pH 2 step; EAP2). Dotted-dashed, medium grey line (EAP2-10) represents the same ethyl acetate phase after the second extraction with aqueous phase at pH 10 (EAP2-10). It was tested in order to elucidate the distribution of bioactivity between the aqueous and ethyl acetate phase. Dotted, light grey line (EAP2-10-2) represents C18-fractions from the third ethyl acetate phase of the second aqueous phase acidified back to pH 2 (EAP2-10-2). Error bars represent standard deviation as propagated through the normalisation to dry weight and averages of absorbance at 580 nm of the respective axenic controls.

However, the notable biofilm induction of the second ethyl acetate phase (EAP2-10) also means that the alkalisation in the second liquid-liquid extraction step yielded very incomplete transfer of the bioactivity into the aqueous phase. It remains unclear whether the chosen liquid-liquid extraction strategy is optimal given the complexity of the Bacteroidetes
DISCUSSION

strain 32 supernatant due to LB medium and bacterial secretions. Less complex media on the other hand yielded much lower strain 32 cell densities (Fig. 3.1, p. 37).

The generally highest bioactivities in the final solid phase extraction step (100% MeOH) indicate that the phases may have been suboptimal for the given chemical properties of the bioactivity. Less hydrophobic materials than C18 and/or more hydrophobic eluents than MeOH should be tested in the future to systematically optimise each step of the S32 supernatant purification. However, the rather equal distribution of the bioactivity into the second aqueous and ethyl acetate phases (AP2-10 and EAP2-10) enabled us to utilise the latter as the end-point for further purification steps. It was initially assumed that the bioactivity was retained in EAP2-10 because the first ethyl acetate extract (EAP2) was neutralised with the alkalised aqueous phase in the second liquid-liquid extraction step. The similarly bioactive AP2-10 fraction, for example, was found to have pH values of around 4.5 instead 10. This likely lowered the transfer of then only partially deproto- nated bioactive compounds from the intermediate EAP2, but even buffering the aqueous phase with NaNCO₃/NaOH at pH 10 did not eliminate this problem. We assume that protonation of the bioactivity enhanced its transfer from aqueous into ethyl acetate phase better than deprotonation assisted the reverse transfer direction. The bioactive second liquid-liquid extract EAP2-10 should therefore be considered for further purification steps, because it was similarly pure as the less accessible third ethyl acetate phase (EAP2-10-2).

Discussion

Our taxonomic placement of Bacteroidetes strain 32 as a Dyadobacter confirms the close relationship to D. ginsengisoli and D. fermentans, which Bruckner et al. [2008] found by BLASTN. Our use of the Ribosomal Database Project (RDP) classification follows the demonstration of Cole et al. [2005] and Wang et al. [2007] that a naïve Bayesian classifier provides a more accurate alignment of closely related ribosomal gene sequences than BLAST’s greedy algorithm [Zhang et al., 2000]. This “initial taxonomic placement” supported our goal of finding recommended, genus-specific growth media for our Bacteroidetes S32. RDP classification and BLAST results together indicate that S32 is either one of the known Dyadobacter species, or a new one in that genus, or the representative of a novel genus that is closely related to Dyadobacter. Because another novel bacterial genus, which utilises carbohydrates from the EPS of diatom biofilms in Lake Constance, was recently sequenced by Rahalkar et al. [2015], S32’s 16S sequence was compared against this Elstera litoralis draft genome. Scores, query cover and identity (599, 81% and 76% respectively) were even lower than for the closest non-Dyadobacter relative in the BLAST search for S32’s 16S sequence (see Suppl. Table 6.1, p. 94). Therefore, our Bacteroidetes strain 32 is not Elstera litoralis. Instead, S32’s growth in the recommended Dyadobacter media was confirmed (see chapter 3, p. 37). Interestingly, no medium was able to sustain notable growth at 37°C, which underscores the environmental origin of S32, as also demonstrated by the variety of climes and isolation sources of other Dyadobacters (see Accession details of Table 6.1 in the NCBI Nucleotide database).

The bioactivity of Bacteroidetes strain 32 supernatant could be tracked through the pH-adjustments and liquid-liquid extractions. The first liquid-liquid extraction step was expected to extract many hydrophobic compounds beside the bioactive one from the LB
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medium. Notable purification was expected in the second step, and the third was added to transfer the bioactivity into an organic solvent again for a more convenient workup. The biofilm-inducing compound(s), however, remain(s) to be identified. This is mostly due to the purities and amounts of extract fractions being too low, but also due to problems with the bioassay. It will be necessary to optimise many aspects of the bioassay, such as shortening the incubation time and simplifying the data collection.

The effectiveness of a bioassay-guided fractionation and purification is supported by the ability of the assay to provide stronger measurable signals upon treatment with purer or more highly concentrated bioactive fractions. The *A. minutissimum* biofilm assay turned out to have a limited ability to do so (see right panel in Fig. 3.3). It was never observed, that concentrated extracts of LB cultures with high S32 cell densities (OD$_{600}$ >0.9) yielded visibly denser biofilms than the co-cultures with Bacteroidetes cells (compare data points “S32” and “AP2-10 LB” in Fig. 3.3 for example) or xenic *A. minutissimum* cultures. This means that possible progress in the purification of bioactive substances of S32 could only be tracked in the limited range between the negative and positive controls. Positive controls with xenic *A. minutissimum* and other LB extract fractions did not induce denser biofilms either, and the diatom was never observed to grow into multi-layered biofilms. Rather, highly concentrated extracts sometimes yielded lower biofilm intensities than less concentrated ones (data not shown). These effects were not consistently reproducible, but they highlight the possibility that *A. minutissimum* reacts to S32’s biofilm inducing compounds in a potentially narrow concentration range, further complicating this bioassay-guided fractionation.

Because no purification or concentration of the bioactive compound(s) of S32 could be achieved, the structure can only be speculated about at this time. One potential candidate group are isomerised and hydroxylated fatty acids, which were shown to occur in a large variety in *Dyadobacter* species before [Chaturvedi, 2005; Baik et al., 2007; Lang et al., 2009], but little is known about their potential secretion. Fatty acid hydroperoxide products have been eluted from a C18 sorbent like we used, but with methyl formate [Graff et al., 1990], which is less polar than MeOH. However, fatty acids have also been identified in the antibacterial defence of diatoms [Findlay & Patil, 1984; Desbois et al., 2008]. This casts the hypothesis that S32’s bioactive compound is a fatty acid derivative into doubt.

It may also be possible that our *Dyadobacter* strain produces pheromone-like substances to interfere with the cell-to-cell signalling of *A. minutissimum*. Oxy lips, L-diproline, and indole-3-acetic acid (IAA) for example are reproductive pheromones in diatoms [Pohnert & Boland, 2002; Gillard et al., 2013; Amin et al., 2015], but it is unknown if or how pheromones may have a function in biofilm formation. Neither biofilm-inducing compounds for diatom-to-diatom communication, nor such compounds of specifically bacterial origin, have been identified to date. IAA could be tested on *A. minutissimum* in a later study, but was not found to induce biofilms (see Fig. 6.4, p. 81). More experiments with pure compounds are complicated by the diversity of hydrophobic, diatom-related compounds, as well as the possibility that the biofilm induction is a result of several chemicals [Byers, 1992].

If the identity of receptor proteins which mediate biofilm formation in our diatom were (or became) known, the following alternative purification strategy could be conducted. Recombinantly produced receptors could be immobilised onto magnetic beads, in order to filter the unknown receptor ligand(s) out of the bacterial supernatant. This concept has been demonstrated in the purification of small molecules from plant extracts and the
identification of ligands for human estrogen receptors [Choi & Breemen, 2008]. In the context of small molecules from Bacteroidetes strain 32, it would first be necessary to identify the receptors in *A. minutissimum*, which mediate the biofilm-induction. This may for example be possible via knock-out libraries of diatom receptors. No such libraries have been generated so far, but several targeted mutagenesis tools have recently been adapted to the diatom model organism *P. tricornutum* [Daboussi et al., 2014; Karas et al., 2015; Weyman et al., 2015]. Meganucleases, nuclear episomes, plasmid delivery via *E. coli* conjugation, or transcription activator-like effector nucleases should be utilised to knock out known diatom receptor proteins. Such knockout mutants could then be screened for a lack of response to bacterial interaction partners. Thus, new receptors could be identified, and subsequently the compounds that mediate the complex chemical interaction of bacteria and diatoms.

**Summary and conclusion**

We have shown that the diatom biofilm-inducing activity of Bacteroidetes strain 32 can be extracted from the bacterium’s acidified supernatant by liquid-liquid extraction and solid-phase extraction. In the latter, the fully methanolic fraction was found to be purest. Methanol simplified the preparation of the extract residues and their application in the bioassay, but introduced the risk of inhibiting growth of the negative control cultures. It did, however, not change the expected biofilm formation patterns of the positive and negative controls. Ultimately, purities and amounts of bioactive fractions that were extracted from the bacterial supernatants were not sufficient to continue with the identification. Moreover, the more than week-long incubation of the bioassay presented in chapter 2 (p. 13) hindered the effective iterative improvement of the fractionation strategy. Therefore, further research should either focus on the metabolic and genetic characterisation of strain 32, or on improving the reproducibility of the bioassay, before it is applicable for the assay-guided purification of S32’s bioactive chemical(s).

**Acknowledgements**

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Chapter 4

A semi-automated, KNIME-based workflow for biofilm assays

Katrin Leinweber$^{1234}$, Silke Müller$^{54}$ & Peter G. Kroth$^4$

Abstract

A current focus of biofilm research is the chemical interaction between microorganisms within the biofilms. Prerequisites for this research are bioassay systems which integrate reliable tools for the planning of experiments with robot-assisted measurements and with rapid data processing. Here, data structures that are both human- and machine readable may be particularly useful. In this report, we present several simplification and robotisation options for an assay of bacteria-induced biofilm formation by the freshwater diatom *Achnanthidium minutissimum*. We also tested several proof-of-concept robotisation methods for pipetting, as well as for measuring the biofilm absorbance directly in the multi-well plates. Furthermore, we exemplify the implementation of an improved data processing workflow for this assay using the Konstanz Information Miner (KNIME), a free and open source data analysis environment. The workflow integrates experiment planning files and absorbance read-out data, towards their automated processing for analysis. Our workflow lead to a substantial reduction of the measurement and data processing workload, while still reproducing previously obtained results in the *A. minutissimum* biofilm assay. The methods, scripts and files we designed are described here, offering adaptable options for other medium-throughput biofilm screenings.

Keywords
diatom, *Achnanthidium minutissimum*, biofilm, medium throughput, bioassay, diatom-bacteria interactions, KNIME

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Introduction

Diatoms (Bacillariophyceae) are a group of highly productive unicellular photoautotrophs and responsible for roughly one fifth of Earth’s primary production [Field et al., 1998; Mann, 1999]. Many diatoms produce and secrete extracellular polymeric substances (EPS) which are a major component of their extracellular polymeric matrices, and which convey motility and substrate adherence [Wetherbee et al., 1998; Wang et al., 2000]. Such aggregates of microbes that are embedded in a matrix of secreted EPS form so called biofilms [Vert et al., 2012]. Biofouling, the colonisation of man-made structures like e.g. ship hulls or underwater constructions by diatoms and other aquatic organisms may causes considerable maintenance costs [Schultz et al., 2010; Wingender & Flemming, 2011].

*Achnanthidium minutissimum* is a benthic pennate diatom that appears cosmopolitan in freshwater habitats which may form biofilms on substrata [Round & Bukhtiyarova, 1996]. Carbohydrate secretion and biofilm formation by *A. minutissimum* can be induced by certain bacteria, but also in the presence of substances secreted by the bacteria, indicating that the bacteria induce or even may control biofilm formation by the diatom [Bruckner et al., 2008, 2011]. In order to quantify biofilm formation as well as to purify and identify bacterial signal substances, a bioassay based on these effects has been established in our laboratory previously [Windler et al., 2015]. This biofilm assay relies on the staining of diatom cells with crystal violet (CV). This compound is also used for staining of Gram-positive bacteria. It is binding to organic polymers such as polysaccharides and peptidoglycans (Popescu & Doyle [1996, Table 2]). Subsequently, the stain can be extracted with ethanol (EtOH), and quantified photometrically. However, this protocol is not automated, and thus proved to be difficult to apply to medium-throughput screenings, e.g. for biofilm-inducing, bacterial infochemicals or biofilm-relevant mutants.

Inspired by other biofilm assay approaches which include automated steps [Stafslien et al., 2006; Buhmann et al., 2012], we aimed to improve the *A. minutissimum* bioassay described above with semi-automated pipetting and absorbance read-out methods. Additionally, we wanted to improve the preparation of sample metadata and biofilm measurement data for analysis, adopting an already widely used tool in nucleotide sequencing and biochemical screening: the Konstanz Information Miner (KNIME; Sundaramurthy et al. [2014]; Steinmetz et al. [2015]). The program is an interactive, visual, modular environment and open platform for the design and execution of data analysis workflows [Berthold et al., 2007]. To the best of our knowledge, no diatom- or biofilm-focussed approach so far is based on KNIME-supported data analyses or shares such workflows. Therefore, the objective of this study is to explain the above-mentioned automation options, and to present compelling evidence for their improvement potential using several examples from a laborious diatom biofilm assay.

Materials and methods

Cultivation conditions

Xenic cultures of *Achnanthidium minutissimum* (Kützing) Czarnecki [1994] were isolated from epilithic biofilms from the littoral zone of Lake Constance, and axenified as described by Windler et al. [2012]. Both xenic and axenic *A. minutissimum* stocks were
cultivated in modified BM (Bacillariophycean medium) in ventilated tissue culture flasks (Sarstedt, Newton, NC, USA). These were stored in a climatised culture room at 16°C, 50% humidity and in a day-night cycle of 12 h at 20-50 \( \text{mol photons m}^{-2} \text{s}^{-1} \) and 12 h of darkness. Cultures were subcultivated monthly by scraping cells off the flask bases and transferring 1 mL of the resulting suspension into fresh flasks with ca. 40 mL BM.

Bacteroidetes strain 32 (S32) was cultivated on agar plates prepared from 50% (v/v) LB [Miller, 1972]. Between monthly transfers to fresh plates, S32 was grown for 5 days at 22°C in darkness, and was then stored at 4-8°C. Liquid S32 cultures were prepared by inoculating 50% LB and shaking at 22°C and 120 rpm for 3-5 days.

**KNIME workflow for data processing**

Due to the incubation time of the diatom biofilms, about two weeks usually passed between preparing the plate layout worksheets and obtaining the measurement results as described in sections *Bioassay experiments* and *Absorbance read-outs of stained and unstained biofilm assays*. In order to link planning documents of an experiment with the final data analyses and results visualisations, we designed a data processing workflow that unifies multiple files for further analysis (Fig. 4.1 and supplemental file KNIME-workflow.tar, p. 95). The workflow was implemented in the Konstanz Information Miner (KNIME) v3.1.1 [Berthold et al., 2007] using freely available functions and nodes, particularly the HCS-Tools extension [Stöter et al., 2013].

Before execution of this workflow, three manual adjustments have to be made. Firstly, file path and name of the layout worksheets in .xlsx format (see Fig. 4.2A and supplemental file plate-layout-template.xlsx, p. 95) were selected for each plate through the respective Load Layout nodes. Secondly, once the corresponding absorbance data files were obtained, they were selected through the respective File Reader nodes. Lastly, the machine-readable export target file has to be defined in CSV Writer.

More plates can be accommodated by inserting more groups of Load Layout, File Reader, Expand Well Position, Joiner and Concatenate nodes into an existing connection between a Joiner and a Concatenate node by copying, pasting and reconnecting the relevant nodes. Thus, the researcher can quickly expand the provided minimal example (see Fig. 4.1) to the desired number of plates, accommodating any number of biological or technical replicates at a time and/or experiment repetitions over time, as long as the tree-like scheme is preserved.

Based on the exported .csv, downstream analysis and visualisation of the KNIME-processed data (Figures 4.3 to 4.5) was conducted in a separate work step in RStudio v0.99 Desktop Open Source Edition with the ggplot2 package v2.0.0 [Wickham, 2009] and R v3.2.3 [R Core Team, 2015].

**Bioassay experiments**

Bioassays were prepared in 48-well plates (#677180, Cellstar; Greiner Bio-One, Frickenhausen, Germany) by scraping both axenic and xenic *A. minutissimum* cultures off the flask bottoms and washing the cells once with fresh BM to remove residues of the spent cultivation medium (see supplemental figure 6.12, p. 96). Axenic cells in this stock suspension were counted in a Thoma chamber in order to calculate the cell concentrations. After extracting chlorophyll in 5% MeOH and 95% acetone according to Jeffrey & Humphrey
from both diatom cultures, their washed suspensions were independently adjusted to \(10^6 \text{cells mL}^{-1}\). For that purpose, both cultures were assumed to contain equal amounts of chlorophyll per cell. This stock suspension of 50 µL (containing \(5 \cdot 10^4\) washed cells) was transferred into each well, as pre-recorded in the worksheets discussed in section Human- and machine-readable data processing.

Treatments were administered within minutes of pipetting the diatom cultures into the multi-well plates. For co-culture experiments of the axenic diatom with S32, suspensions of the bacterial cells were prepared by centrifuging the LB liquid cultures at \(5-10 \cdot 10^3\) g for 5-10 min and washing them with BM supplemented with 10 mM glucose (glcBM). The washed bacterial cells were resuspended in BM to an OD of 0.1 and 5 µL of that suspension was added to the wells with the axenic \textit{A. minutissimum} cultures. For supernatant experiments, different volumes of sterile-filtered bacterial supernatant were added to the wells. This supernatant was harvested from S32 cultures in glcBM and filtered through 0.2 µm pores (Sarstedt AG, Nümbrecht, Germany). In either case, each well was filled with fresh BM to a final volume of 500 µL. Negative control wells contained untreated axenic \textit{A. minutissimum}, while xenic cultures served as biofilm-positive controls. Bioassay plates were sealed with Parafilm (Bemis, Neenah, WI, USA) and cultivated for 10-12 d as described for the stock cultures in section Cultivation conditions.

Robotised biofilm quantification

We evaluated the utility of robotised pipetting for the removal of non-adherent cells and medium, as well as for the staining of the remaining biofilms with crystal violet (CV) as visualised in Fig. 4.2C. VIALINK Pipette Management Software was used to program custom scripts. Repeat Dispense scripts were programmed on a 1250 µL Viaflo Electronic Pipette. The latter was used with six 1250 µL pipette tips for all pipetting steps, along the long axis of the 48-well plate. We tested using eight tips and pipetting along the short axis of the plate, but found that they did not reliably align with the wells. Both script types (see Table 4.1) were executed with the pipette connected to the Viaflo ASSIST robot (INTEGRA Biosciences AG, Switzerland).

Table 4.1: Overview of electronic pipetting scripts executed by the Viaflo ASSIST robot

<table>
<thead>
<tr>
<th>Step</th>
<th>Mode</th>
<th>Script task</th>
<th>Height/Z</th>
<th>Speed</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>custom</td>
<td>mix &amp; remove cells</td>
<td>54.5 mm</td>
<td>7</td>
<td>yes</td>
</tr>
<tr>
<td>2.</td>
<td>repeat disp.</td>
<td>add 200 µL CV</td>
<td>10 mm</td>
<td>3</td>
<td>no</td>
</tr>
<tr>
<td>3.</td>
<td>custom</td>
<td>remove CV</td>
<td>54.5 mm</td>
<td>3</td>
<td>yes</td>
</tr>
<tr>
<td>4.</td>
<td>repeat disp.</td>
<td>add 1 mL H(2)O</td>
<td>10 mm</td>
<td>5</td>
<td>no</td>
</tr>
<tr>
<td>5.</td>
<td>custom</td>
<td>remove H(2)O</td>
<td>54.5 mm</td>
<td>8</td>
<td>yes</td>
</tr>
</tbody>
</table>

The crucial, optimisable step for removing non-adherent cells is the mixing step. For our
A. minutissimum cultures, mixing was optimised in terms of immersion depth (54.5 mm; see Table 4.1), mixing volume (400 µL), speed (7) and cycles (7) at room temperature, so that axenic cultures were resuspended while the xenic biofilms were not disturbed. These settings have to be adjusted for other organisms, in order to accommodate different biofilm thicknesses, strengths of adherence, etc.

The plate reader device Tecan Infinite F500 (Tecan Group Ltd., Switzerland) was used at room temperature to measure biofilm absorbances. For that purpose, a custom method (see supplemental file Magellan-readout.mth, p. 95) was implemented in the Magellan data analysis software v7.1. It took absorbance measurements at 16 evenly spread points in a filled circle with a 1.7 mm distance to the well edge. The absorbances of unstained biofilms were read out at 630, 650 and 750 nm. These wavelengths were chosen according to Jeffrey & Humphrey [1975] for the chlorophyll c absorbance maximum (630 nm), for a compromise between the chl a maximum and an available filter (650 nm), as well as for the light scattering due to cells (750 nm). After measuring the absorbances of the unstained biofilms, they were incubated for 1 min with 200 µL of a 1:100 dilution of the crystal violet (CV) staining reagent described by Kaplan & Fine [2002]. The CV solution was removed by pipetting as described above. Afterwards, sterile-filtered tap water was added to the wells for washing, and removed after 1 min. The plate-reader was used again to measure the absorbance at 580 nm. Finally, the Magellan method ensured the data export into machine-readable .asc files for subsequent input into the KNIME workflow.

For comparison of the above described robotised biofilm quantification with the manual protocol established by Windler et al. [2015], CV was extracted from the wells after plate-reading. This was done by pipetting 1 mL EtOH up and down several times and the resulting CV solution was transferred into a quartz cuvette. The absorbance was measured at 580 nm in an Ultrospec 2100 pro UV/visible spectrophotometer (Biochrom Ltd. Cambridge, UK).

Results and discussion

Human- and machine-readable data processing

Regarding our use of the plate layout worksheets and KNIME, we found that using such software for managing experimental workflows is advantageous [Tiwari & Sekhar, 2007; Wyres et al., 2014]. This results from the data processing being reviewable independently [Ghosh et al., 2013], reproduced by other researchers, as well as prepared and tested with example data or initial measurement data. We also found KNIME useful for tracking the progress of experiments because all nodes indicate their status (traffic light symbols in Fig. 4.1). This becomes particularly useful for complex experiments during which replicate data is obtained at different times and/or after long incubation times (e.g. seasonal repetitions, see Sekar et al. [2004]).

We implemented and tested automation options for pipetting, measurement and data processing in the context of the Achnanthidium minutissimum bioassay, in order to make it applicable for medium-throughput screenings. For the tests, we prepared biofilm-negative and -positive samples from axenic and xenic A. minutissimum, as well as the Bacteroidetes strain 32 (S32). These work steps are summarised in the upper part of figure 4.2, while the lower part covers the newly developed robotisation and data processing methods presented below.
Figure 4.1: KNIME workflow example for the integration of sample metadata and measurement results for analysis & visualisation. The green indicator below each node indicates that the entire workflow has been successfully executed. See supplemental file KNIME-workflow.tar, p. 95 for an importable copy of this workflow, which serves as an interactive tutorial for the methodology presented in this article.
RESULTS AND DISCUSSION

Figure 4.2: Overview of semi-automated biofilm assay from metadata tables (A) to measurement workflow (C). Hexagonal boxes indicate manual steps of biofilm assay procedure. Rounded boxes represent robotised steps. Dashed, grey-lined boxes represent main sections of the biofilm assay protocol. A: Metadata tables in .xlsx worksheet are used to plan the layout of samples on the multi-well plate (see supplemental file plate-layout-template.xlsx, p. 95). Table headings represent factors (cell_culture & treatment), whose levels (respectively, ax, co & xen, as well as NA for controls & S32 for co-cultures) fill the plate-congruent coordinate systems of the tables. Grey cells indicate bases for formulas, which help to rapidly fill repetitive tables with for example technical replicates or dilution calculations. B: Photo of incubated plate, ready for removal of non-adherent cells and absorbance measurement. C: Robotised measurement preparation (see Table 4.1), absorbance read-out and data processing from biofilm assay plates (see supplemental figure 6.12 and files Magellan-readout.mth, KNIME-workflow.tar and plot-KNIME-output.R, p. 95).

Preparing plate layouts in .xlsx-compatible programs enabled the calculation and planning of dilution series, and even randomisations. However, the potential benefit of
randomised sample placement should be evaluated on a case-by-case basis against the increased workload and the risk of pipetting errors during manual inoculation. This risk can be mitigated by keeping dilution series in their inherent order, but pipetting them in alternating directions across the plate (pseudo-randomisation). The worksheets should obviously be prepared before the inoculation, in order to be useful as pipetting guides at the bench. However, they can be updated at any time to reflect pipetting errors, changes of the experimental plan, or the sample sets, etc.

The KNIME workflow may be improved by integrating such scripts, so that data analysis and visualisation can be started immediately with the first absorbance data file. Because inputting the paths of the corresponding layout worksheets and absorbance data files into the nodes is repetitive, it may be desirable to automate the detection and pairing of corresponding files in large numbers. Such an automation may be possible using KNIME's Read XLS Sheet Names, StringManipulation, flow-variables and loops (personal communication, Patrick Winter).

**Robotised biofilm quantification**

Regarding the robotised liquid removal steps, we found that they could not be conducted reliably at this immersion depth, because notable residue volumes remained in the wells. Immersing the pipette tips further risked scratching the biofilms, so we instead used the electronic pipettes manually, tilting the tips into the well edges. Despite this manual removal of liquids, the time requirement and work intensity per plate could be reduced considerably from ca. 30-45 min of intense manual pipetting Windler et al. [2015] to ca. 10-15 min of intermittent electronic pipetting and device operations.

A read-out of the EtOH-filled wells after CV extraction was possible, but similarly to a report by Rasmussen & Østgaard [2001] (Discussion | Experimental Systems), the meniscus of the solution distorted the measurements in a ring-shaped area around to the well edges. As smaller well sizes may exacerbate this problem, we refrained from using 96-well plates. Manual removal of residual liquid with finer pipette tips, or a separate drying step might alleviate this risk, but would extend the time for measurements.

Our workflow can be adopted easily to other organisms and biofilm assays by adjusting the plate-reading method (supplemental file Viaflo-scripts.tar; particularly the number of the measurements, as well as the chosen wavelengths) in Tecan’s Magellan software. One potential improvement is the randomisation of the measurement positions in the wells [Silzel et al., 2005], but this was not yet available in our Magellan version.

The bottleneck of this workflow is the number of samples and plates that can reasonably be inoculated manually. Dispensing Bacillariophyceae medium (BM) and *A. minutissimum* with a stepper or multi-channel pipette requires only a few minutes per 48-well plate, but the application of different treatments (e.g. S32 co-cultivation or its supernatant extracts, or compounds at different dilutions, etc.) easily multiplied that time per plate. To remove this bottleneck, further robotisation protocols similar to those reported by Tillich et al. [2014] are required. For that purpose it will be necessary to either add control cultures for that protocol’s semi-sterile nature, or to optimise it towards fully sterile conditions. As a next step, a robotised *A. minutissimum* and S32 co-cultivation system could be connected to high-resolution mass spectrometry in order to elucidate the nature of biofilm-inducing substance [Tillich et al., 2014; Windler et al., 2015].
Absorbance read-outs of stained and unstained biofilm assays

The initial proof-of-concept of the above-described measurement protocol was conducted with non-adherent, axenic (ax) and biofilm-forming, xenic (xen) A. minutissimum cultures (Fig. 4.3). Using the plate reader resulted in lower absolute absorbances for both culture types. The spread of data read-outs from axenic replicates was about half of those from the EtOH- and photometer-based measurements. For xenic biofilms, the spread was only 25% smaller, but the plate reader measured lower absorbances. Nevertheless, the absorbance read-outs still were clearly distinct between axenic and xenic samples.

The EtOH- and photometer-based method by Windler et al. [2015] resolved the differences of biofilm intensities better. However, xenic replicates with strong deviation below the median absorbance could result from drying of the biofilms before staining or before the extraction of the stain with EtOH. Axenic replicates with strong deviation above the median absorbance could readily result from small crystal violet residues getting carried into the EtOH extraction. Therefore, the EtOH- and photometer-based method risks false measurements in both controls. This previous method is only feasible for the quantification of small sample sets. The faster plate-reading was less error-prone and more reproducible. It still allows the EtOH- and photometer-based method to be appended in case additional verification of unclear read-outs is required.

The next step of our proof-of-concept deals with the detection of unstained biofilms at 630, 650 and 750 nm (Fig. 4.4). Median absorbances were significantly distinct between the axenic, non-adherent control and the biofilm-forming co- and xenic cultures in any case. The total spreads of data from the axenic controls overlapped only slightly with those of the two biofilm-positives sample types (co- and xenic culture). The median absorbances after staining with CV were more distinct, and the data spreads did not overlap, although those of the biofilm samples were larger. In summary, the medians and spreads of data show that the distinctiveness of unstained biofilm-free and -containing wells was reflected by the absorbances on three different wavelengths as well as by the CV-specific wavelength.

The similarity of biofilm absorbances read-outs at 630, 650 and 750 nm confirms other biofilm measurements conducted at wavelengths of 680 nm [Lefaive & Ten-Hage, 2010] and 600 nm [Stafslien et al., 2006]. The latter study also involved staining with CV of bacterial biofilms in multi-well plates, in order to test combinatorially produced surface coatings for anti-fouling capabilities. The similarity of technical set-ups suggests that our KNIME-based workflow can be applied beyond diatom biofilm research. In another photometric approach Buhmann et al. [2012] monitored biofilm formation of the diatom Planothidium sp. in a sterile incubator. They detected the growth of unstained biofilms by attenuation of the visible light spectrum around a peak of 600 nm in real-time. The common result of those cited with our study is that diatom and bacterial biofilms can be detected between 580 and 680 nm. This suggest that any wavelengths within this range is suitable. Moreover, the cited biofilm assays were conducted with less than ten replicates. Our workflow helps to handle more biological and/or technical replicates at a time, experiment repetitions over time, and/or repetitions by different researchers. All these are key to the statistical power of studies and help to reduce the risk of false research findings [Ioannidis, 2005], but increase the workload considerably. Our workflow decouples the number of replicates and repetitions from the data processing and analysis workload.

The lower absolute absorbances of unstained biofilms at 630, 650 and 750 nm (Fig. 4.4) can be explained by the detection of only adherent cells. CV stains EPS structures like stalks and capsules in addition to the cellular polymeric structures [Popescu & Doyle, 1996],
Figure 4.3: Comparison of photometer- and plate-reader-based absorbance measurements of CV-stained *A. minutissimum* biofilms. Biofilms stained with CV were first measured in the wells with a plate reader (left facet). Afterwards CV was extracted with EtOH, transferred into photometric cuvettes and re-measured in a spectrophotometer (right facet). Axenic (ax) and xenic (xen) wells represent the negative and positive controls in this bioassay (non-adherent and adherent cells, respectively; *N* = 36). Boxes represent the first and third quartiles. Whiskers extend to the lowest and highest value that lies within 1.5-fold of the inter-quartile range (IQR). Black dots are extreme values that lie outside the IQR. Black center lines represent medians. Notches indicated the range of $1.58 \cdot \text{IQR}/\sqrt{N}$ (ca. 95% confidence interval) around the medians. For a more detailed explanation of box-and-whisker plots and their variants, we recommend reading McGill et al. [1978]. See also supplemental file `plot-Tecan-figures.tar`, p. 95 for R code and data used to plot this and the following figures.
and thus contributes to the high absolute absorbances at 580 nm. EPS are important for the surface attachment of microbes and are a logical requirement for biofilm formation [Wetherbee et al., 1998; Wang et al., 2000]. Our protocol supports the staining where necessary, but the elimination of CV staining from workflows does not prohibit the biofilm detection. CV-free workflows can be desirable, because the stain may increase the risk of false-positives, and is classified as toxic to aquatic life [National Center for Biotechnology Information & U. S. National Library of Medicine, 2015].

As a final assessment of our proof-of-concept, we tested the biofilm induction in axenic A. minutissimum by addition of sterile-filtered S32 supernatant. We used the same measurement set-up as described for figure 4.4. All absorbance curves peak between 10-50% (v/v) supernatant (Fig. 4.5). Absorbances of unstained biofilms measured by plate-reader at 630, 650 and 750 nm had lower absolute differences. At those wavelengths, the unstained biofilms had the absorbance maxima at 25% supernatant. After staining with CV and measurement at 580 nm, the maximum shifted to the range of 25-50%.

The shapes of all biofilm-induction curves (Fig. 4.5 agree with analogous curves determined previously with the EtOH-extraction protocol (Windler et al. [2015], Fig. 3A therein). This demonstrates that the less time-consuming protocol proposed in the present study is able to detect differently strong, unstained biofilms, as already discussed for the light-attenuation-based evidence reported by Buhmann et al. [2012]. In addition to the faster, non-invasive biofilm detection, our workflow leaves two opportunities to increase the absorbance resolution for unclear samples: (1) Read-out of stained biofilms can easily be integrated; (2) EtOH extraction of the stain and photometric measurement can be appended as well.

To summarise, the three proof-of-concept steps confirmed the results of previous studies and highlighted several simplification and automation options. These options are applica-
Figure 4.5: Detection of differently strong *A. minutissimum* biofilms before and after staining with CV. Bacteroidetes strain 32 supernatant was centrifuged and sterile-filtered from three 7 d old glcBM liquid cultures with OD$_{600}$ of 0.25. Biofilm induction was highest around 25-50% spent bacterial medium (v/v) as shown in figure 3A of Windler et al. [2015].

...ble for biofilm assays in general, and specifically enhance the utility of *A. minutissimum* in environmental monitoring. This is relevant, because this diatom has been used previously as a bioindicator of heavy metal pollution [Falasco et al., 2009; Cantonati et al., 2014]. That application relied on the detection of frustule deformations – an approach that has been questioned by Lavoie et al. [2012] who observed the valves often settling in girdle view, which hindered the quantification of frustule deformations in this diatom species. Using *A. minutissimum* as an example, we present the simpler, robotised proxy measurement of the presence or absence of cells as a means to detect biofilms. Thus, we demonstrate here an improved *in vitro* variant of *in situ* “stable platforms”, which Amin et al. [2012] recommended for the study of diatom-bacteria interactions.

**Summary and conclusion**

In this report, we present several simplification and robotisation options for an assay of bacteria-induced biofilm formation by the freshwater diatom *Achnanthidium minutissimum*. We also tested several proof-of-concept robotisation methods for pipetting, as well as for measuring the biofilm absorbance directly in the multi-well plates. Furthermore, we exemplify the implementation of an improved data processing workflow for this assay using the Konstanz Information Miner (KNIME), a free and open source data analysis environment. The workflow integrates experiment planning files and absorbance read-out data, towards their automated processing for analysis.
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Chapter 5

Capsules of the diatom
*Achnanthidium minutissimum*

arise from fibrillar precursors and foster attachment of bacteria

Katrin Leinweber\(^1\)\(^2\)\(^3\) \& Peter G. Kroth\(^4\)

Abstract

*A. minutissimum* is a benthic freshwater diatom that forms biofilms on submerged surfaces in aquatic environments. Within these biofilms, *A. minutissimum* cells produce extracellular structures which facilitate substrate adhesion, such as stalks and capsules. Both consist of extracellular polymeric substance (EPS), but the microstructure and development stages of the capsules are so far unknown, despite a number of hypotheses about their function, including attachment and protection. We coupled scanning electron microscopy (SEM) to bright-field microscopy (BFM) and found that *A. minutissimum* capsules mostly possess an unstructured surface. However, capsule material that was mechanically stressed by being stretched between or around cells displayed fibrillar substructures. Fibrils were also found on the frustules of non-encapsulated cells, implicating that *A. minutissimum* capsules may develop from fibrillar precursors. Energy-dispersive X-ray (EDX) spectroscopy revealed that the capsule material does not contain silicon, distinguishing it from the frustule material. We furthermore show that bacteria preferentially attach to capsules, instead of non-encapsulated *A. minutissimum* cells, which supports the idea that capsules mediate diatom-bacteria interactions.

**Keywords**

diatom, biofilm, scanning electron microscopy, energy-dispersive x-ray spectroscopy, diatom-bacteria interaction

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Introduction

Diatoms (Bacillariophyceae) are among the most productive photoautotrophic, aquatic microorganisms. They contribute an estimated 40-45% to the net primary production (NPP) of the oceans [Mann, 1999], which themselves contribute approx. 45-50% to the global NPP [Field et al., 1998]. Additionally, diatoms are important for the biogeochemical cycling of silicon, due to their ornate cell walls. These are called “frustules” and are composed of biomineralised silica [Bradbury, 2004]. Cell division includes the separation of the two frustule parts (“thecae”) along a “girdle” region. Each daughter cell then complements its inherited epitheca with a newly synthesised, smaller hypotheca. Within these thecae, slits (called “raphes”) and pores may be present, facilitating the secretion of extracellular polymeric substances (EPS; Wetherbee et al. [1998]; Wang et al. [2000]). This in turn conveys substrate attachment and motility to benthic diatoms, which often form biofilms with other photoautotrophic algae, as well as heterotrophic bacteria [Buhmann et al., 2012].

The diatom *A. minutissimum* (Kützing) Czarnecki [1994] is a cosmopolitan freshwater diatom [Round & Bukhtiyarova, 1996] that is also found in the littoral zone of Lake Constance. It represents a dominant species complex of early colonisers [Johnson et al., 1997; Potapova & Hamilton, 2007], forming epilithic biofilms in association with a variety of satellite bacteria [Bahulikar, 2006]. Such bacteria as well as their spent media have been shown to increase the secretion of extracellular polymeric substances (EPS) like carbohydrates by *A. minutissimum* [Bruckner et al., 2011]. Additionally, growth as well as secretion of proteins and dissolved free amino acids was reportedly influenced in the presence of bacteria [Bruckner et al., 2008, 2011]. Analogous to the rhizosphere of terrestrial environments (composed largely of fungi and bacteria associated with plant roots), a “phycosphere” has been defined as the space surrounding algal cells including the multitude of inter-kingdom interactions between bacteria and algae [Bell et al., 1974; Amin et al., 2012].

EPS secretion is of ecological relevance, contributing for instance to the stabilisation of sediments [Cyr & Morton, 2006; Lubarsky et al., 2010]. Studying biofilm formation can therefore assist in the understanding of shore and coastline erosion as a result of climate-related changes (see section 3.2.1 of Widdows & Brinsley [2002] plus references therein). At the same time diatom settlement is one of the major causes of biofouling of man-made machinery in aquatic applications [Molino & Wetherbee, 2008].

*A. minutissimum* is an excellent model for studying diatom biofilms, because this alga is abundant *in natura* [Patrick & Reimer, 1966; Krammer & Lange-Bertalot, 1991] and can be cultivated in the laboratory both as “xenic” biofilms [Myklestad et al., 1989] and “axenic” suspension cultures. Xenic cultures contain bacteria from the diatom’s natural habitat. Removal of these bacteria is possible (for example by antibiotic treatment) and yields viable axenic cultures [Bruckner & Kroth, 2009; Windler et al., 2012]. Bacteria-free cultures allow the establishment of bioassays in order to study the interactions between diatoms and bacteria, although potentially unwelcome long-term effects have to be taken into account. For example, axenic cultivation can lead to a reduction of average cell size and to frustule deformations [MacDonald, 1869; Pfister, 1871; Geitler, 1932; Windler et al., 2014]. However, such deformations also occur naturally and can be used as stress markers, as well as water quality indicators [Morin et al., 2008; Falasco et al., 2009; Cantonati et al., 2014].
A. minutissimum forms biofilms and extracellular structures like stalks. These structures have been defined as “unidirectionally deposited, multi-layered structures attaching cells to substrata” [Hoagland et al., 1993]. Stalks have been investigated previously by transmission electron microscopy and biochemical techniques to elucidate their structural morphology and chemical composition [Daniel et al., 1987]. Additionally, a phase model of diatom adhesion involving stalks has been developed [Wang et al., 1997]: Stalks may protrude from so called “basal pads” of aggregated EPS at the apical valve faces within hours to a few days, thus elevating the cells above the substrate. Capsule formation in A. minutissimum biofilms occurs later in the stationary phase, is possibly triggered by bacterial influences, and may cement diatom attachment [Windler et al., 2015]. That study also found that axenic A. minutissimum cultures mostly secrete soluble carbohydrates while the insoluble carbohydrates in xenic cultures were positively correlated with the appearance of capsules.

Diatom capsules have puzzled phycologists for a long time and their potential physiological and ecological function have elicited a variety of hypotheses [Lewin, 1955; Geitler, 1977]. For example, capsules have been proposed to participate in locomotion, flotation, attachment, waste removal, catchment of inorganic nutrients, storage of polysaccharides, sexual reproduction, as well as protection against grazing and dehydration. More recently, it was demonstrated that capsule formation is dependent on at least “a certain minimum light intensity”, sparking the idea that capsules might serve as an additional polysaccharide storage pool, once intracellular capacities are saturated [Staats et al., 2000].

Diatom capsules and other extracellular material have been characterised biochemically, while electron microscopy has enabled highly detailed morphological characterisations of diatom frustules [Toyoda et al., 2005, 2006; Potapova & Hamilton, 2007]. Additionally, atomic force microscopy (AFM) revealed many mechanical properties of the extracellular polymers of some diatoms [Crawford et al., 2001; Higgins et al., 2003; Willis et al., 2013]. In the present study, scanning electron microscopy (SEM), as well as energy-dispersive X-ray (EDX) spectroscopy were employed to analyse the microstructure and development stages of A. minutissimum capsules in order to further develop this species as a model system for diatom-bacteria interactions, and to elucidate one aspect of the complex interactions of diatoms and other microorganisms.

Materials and methods

Cultivation conditions

A. minutissimum (Kützing) Czarnecki [1994] was isolated from photoautotrophic epilithic biofilms of Lake Constance as previously described [Windler et al., 2012]. Stock cultures were grown in cell culture flasks with ventilation caps (Sarstedt, Newton USA) filled with modified liquid Bacillariophycean Medium (BM; Schlösser [1994]; Windler et al. [2012]) in two different culture states: either with co-isolated bacteria (“xenic”), or “axenic” after their removal [Windler et al., 2012]. Monthly, these stock cultures were scraped off the flask bases and sub-cultured in new BM.

Biofilms were grown directly on SEM sample carriers by the following procedure: Sample carrier disks of ca. 1 cm in diameter were punched from Thermanox tissue culture cover slips (Miles Laboratories Inc., USA). Thermanox material has two different sides, therefore care was taken to always store and handle the disks right-side-up. They were
sterilised by immersion in 70% isopropanol (v/v in H2O) over night and subsequently irradiated with UV light for 2 h in a laminar flow cabinet. One sterile disk was placed into each well of 6-well plates (Sarstedt, USA, order number 83.1839.500) and covered with 3 to 5 mL BM. Culture wells were inoculated with 5 · 10⁵ to 10⁶ A. minutissimum cells from the stock cultures after those were checked to be axenic or xenic. Axenicity was confirmed by SYBR Green staining and observation under a BX51 (Olympus, USA) bright-field fluorescence microscope using GFP fluorescence filters. Well plates were sealed with Parafilm and incubated at 16°C under an illumination regime of 12 h dark and 12 h light at 20-50 \(\mu\text{mol photons m}^{-2}\text{s}\) for 11 to 31 days.

Crystal violet (CV) staining and bright-field microscopy

Thermanox disks were removed from stationary cultures after 11 to 31 days with inverted (“soldering” or “cover glass”) forceps (Hammacher, Germany) and rinsed with 1 mL sterile-filtered tap water. A Gram-staining protocol adapted from Kaplan & Fine [2002] was applied to visualise adherent cells and their extracellular polymeric structures as follows: A droplet of 200 µL solution of 0.02% CV in sterile filtered tap water was applied onto the disk for 1-2 min, which was held suspended by forceps. Disks were rinsed with 1-3 mL water, until the run-off no longer contained visible CV. In order to find the same cell clusters in both microscopic approaches, pointing or encircling scratches were made into disk surfaces.

Disks were placed on moistened glass slides and moistened additionally with 20 µL sterile-filtered tap water. Cover slips were applied carefully and marked regions were observed under a BX51 (Olympus, USA) bright-field fluorescence microscope using chl fluorescence filters. Images of these areas at various magnifications were taken with AxioCams MRm (fluorescence and grey-scale images) and MRc (colour) using AxioVision software (Zeiss, Germany).

Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) spectroscopy

Diatom cells were fixed on Thermanox disks by incubation in a mixture of 2% glutaraldehyde, 10 mM CaCl₂ and 10 mM MgCl₂ in 0.1 M sodium cacodylate buffer at pH 7 and room temperature (RT) for 2 h. Dehydration was conducted first with 30% and 50% EtOH, at RT for 2 h each, followed by 70% EtOH at 4°C over night, 90% EtOH at RT for 2 h and finally with 96% and 100% EtOH twice for 1 h each. Critical point drying in CO₂ followed (Balzers CPD030, Liechtenstein) and samples were finally sputtered with gold (Au) and palladium (Pd) to a thickness of 5 nm (Balzers SCD030, Liechtenstein).

After fixation, dehydration and Au/Pd-sputtering, the biofilm-covered Thermanox disks were imaged with a Zeiss AURIGA scanning electron microscope, controlled with the SmartSEM software v.05.04.05.00. The elemental composition of samples was analysed by energy-dispersive X-ray (EDX) spectroscopy. Samples were excited with the AURIGA’s electron beam at 10 kV and the emitted X-rays (of specific energy levels due to the elemental electron configuration) were recorded with an X-Max²⁰ detector and the INCA software v4.15 (Oxford Instruments).
Bacteria counting and data visualisation

Bacteria (rod-shaped particles) on fully visible *A. minutissimum* valve faces were counted in scanning electron micrographs. Valve faces were classified into frustules and capsules, depending on whether pores were visible or completely disappeared under a layer of capsule material. Diatom cells with partial encapsulation were not included in the counting, and neither were bacteria cells which attached to the girdle bands of diatom cells.

ImageJ v1.46r with the Cell Counter plug-in v2010/12/07 was used to count diatoms and bacteria cells. This data was evaluated and visualised with R v3.1.1 (language and environment for statistical computing; R Core Team [2015]), ggplot2 v1.0.0 [Wickham, 2009] and RStudio v0.98 Desktop Open Source Edition.

Results and discussion

For BFM and SEM observations, we cultured *A. minutissimum* xenically and axenically on Thermanox disks. After incubation periods of 11 to 31 days, the disk surfaces in xenic cultures were densely covered by a mono-layer of *A. minutissimum* cells (Fig. 5.1). This biofilm was visible by eye as a light greenish-brown colouration on the substrate disks after removal from the medium. Staining with the dye CV and subsequent bright-field microscopy showed that large portions of the diatom cells were surrounded by capsules.

Figure 5.1: Crystal violet (CV) stained capsules (grey ovals) in xenic *A. minutissimum* biofilm (scale bar: 20 µm). Micrograph depicts 11 days old culture and is a merge of the chl fluorescence channel (red; indicating diatom cells) and the bright-field image (grey). Some mature capsules are marked with arrows. Bright spots within diatom cells are lipid bodies. Bacteria are visible as light and dark speckles around and in between the diatom cells.
In contrast, axenic *A. minutissimum* cells did not form biofilms, so that even careful rinsing left much fewer cells attached to the disks and thus available for SEM analysis. This observation is in agreement with studies that utilised other growth substrates to compare biofilm formation by axenic and xenic diatom cultures. By measuring chl concentrations, the possibility that axenic cells might simply be less proliferate was excluded [Windler et al., 2015]. Xenic *A. minutissimum* cultures on the other hand have also been found to develop biofilms on glass beads as well as in plastic multi-well plates [Lubarsky et al., 2010; Windler et al., 2015]. Our results demonstrate, that xenic biofilms of *A. minutissimum* can also be grown on Thermanox disks, enabling direct preparation for electron microscopy of native biofilm samples.

**Identification of *A. minutissimum* capsule microstructures**

In order to correlate the hydrated *A. minutissimum* capsules visible in light microscopy to their dehydrated appearance in SEM, areas were marked by scratches on the CV stained disks and cells of interest were identified by BFM. Subsequently, the same areas and cells were found again in the SEM (Fig. 5.2). The same technique was successfully applied to axenic cultures, despite the lower prevalence of adherent cells (Suppl. Fig. 6.13, p. 97).

![Identification of *A. minutissimum* capsules (asterisks) by successive observation of cell clusters by first bright-field and then scanning electron microscopy of xenic biofilm (scale bars: 5 µm). A: Bright-field micrograph of CV stained, 31 days old culture. Encapsulated cells (asterisks) are strongly stained, while weak staining indicates few extracellular polymeric substances (EPS) on the frustule surfaces. B: Scanning electron micrograph of the same cell cluster. Encapsulated cells (asterisks) are surrounded by an opaque material. Frustule pores are visible on cells that did not possess a capsule in the hydrated biofilm. Note also the unequal distribution of bacteria cells on capsules versus non-encapsulated frustules.](image)

In BFM, the CV stained capsules were visible as voluminous, rounded structures around most of the cells. As extracellular polymeric structures in the genus *Achnanthidium* are composed mostly of carbohydrates [Wustman et al., 1998; Windler et al., 2015], strong hydration in the native biofilm is likely the source of this appearance of the capsules. In the SEM, we were able to distinguish two types of *A. minutissimum* cells in xenic biofilms already at low magnifications: cells with pores in their frustules still visible, and cells covered by an apparently unstructured material masking the pores.
The frustules of non-encapsulated xenic, as well as axenic *A. minutissimum* cells appeared identical to those from scanning electron micrographs shown in previous studies [Mayama & Kobayasi, 1989; Potapova & Hamilton, 2007; Hlúbiková et al., 2011]. The low prevalence of raphes in our images is most likely due to their orientation towards the substrate for mucilage secretion [Gordon & Drum, 1970; Wetherbee et al., 1998]. Natural attachment and orientation of cells on our biofilm disks was retained because we did not employ harsh preparation techniques, such as boiling the diatom cells in sulfuric acid [Mayama & Kobayasi, 1989]. Such harsh treatments are designed to prepare only frustules and in our case would likely have resulted in cell detachment from the growth substrate, as well as random orientation on the SEM sample carrier. Instead, we utilised the SEM sample carrier disks directly as growth substrates for the biofilms.

![Figure 5.3](image)

**Figure 5.3: Comparison of microstructures on *A. minutissimum* cell surfaces in a xenic biofilm.** A (scale bar: 2 µm): Capsule material is sometimes stretched between cells and/or towards the substrate (arrows). Culture was 11 days old at the time of fixation for SEM. Asterisks denote magnified areas B and C. B (scale bar: 1 µm): Non-encapsulated cells possess a fibrillar mesh of varying degrees of density. Frustule pores are only partially covered and in some cases, fibrils stick out from the frustule. C (scale bar: 1 µm): Encapsulated cells are completely covered with a material of slightly granular structure, but lacking clearly discernible features.

The SEM images in Fig. 5.3 show that the capsule material appears to be unstructured or slightly granular, resembling the “adhering film and tube” of *Cymbella microcephala* and *Cymbella prostrata* reported in figures 31 and 32 of Hoagland et al. [1993] and also the shaft ultra-structure of the marine diatom *Achnanthes longipes* displayed in figure 8 of Wang et al. [2000].

In addition to covering the cells, the capsule material also had sheet-like structures (arrows in Fig. 5.3A) where it was stretched between *A. minutissimum* cells and the anchoring points on the substrate. This pattern is most likely due to dehydration during SEM sample preparation [Hoagland et al., 1993]. Due to fixation of the samples prior to drying, the hydrated capsules most likely shrank in their entirety.
Fibrillar precursors may give rise to *A. minutissimum* capsules

Closer inspection provided many examples that the frustules of non-encapsulated xenic *A. minutissimum* cells were not completely free of extracellular polymeric substances (EPS). Instead, they were covered by a mesh of fibrils (Fig. 5.3B), arranged mostly around the frustule pores, sometimes crossing them and sometimes sticking out. The average diameter of these fibrils was about 45±9 nm. The fibrils were rarely observed to be secreted through the pores, although these were found to be large enough (from 60 to 140 nm in diameter), showing round to elongated shapes. Fibrils were generally longer than the diameter of pores, but quantification was not performed because branching and interweaving of the fibrils made it impossible to determine the respective beginnings or ends. To the best of our knowledge, this is the first report of frustule-attached fibril structures in freshwater diatoms. Similarly structured, thinner fibrils were reported previously only for marine diatoms [Bosak et al., 2012].

Fibrillar meshes of varying densities were detected in both axenic and xenic cultures, but only the latter also contained capsules. Axenic cultures appeared to contain more cells with few, short fibrils (Suppl. Fig. 6.14, p. 97), which may initiate surface attachment of cells in both culture types. However, only in xenic cultures, bacteria could induce the secretion of firmer EPS structures [Bruckner et al., 2011], and thus substrate adhesion, in the majority of cells.

Figure 5.4: Scanning electron micrographs of terminal parts of *A. minutissimum* cells at potentially different encapsulation stages within xenic biofilms (scale bars: 1 µm). Fibrillar meshes (A) may form capsule material (C) by denser growth and cross-linking of fibrils (B). Depicted samples were taken from stationary, 11 to 31 days old cultures.

Furthermore, we found intermediate stages between the fibrillar meshes that covered the frustule surface only partially and the complete encapsulation with apparently unstructured material (Fig. 5.4). The similarity in surface structure suggests a relation of fibrils and capsules. The disordered arrangement of fibrils shown in figure 5.4A and B is also a feature of the unstructured capsule material shown in figure 5.4C. In it, no particular order of the slightly granular substructures is discernible either. This visual impression suggests that the fibrillar meshes on *A. minutissimum* cells might be precursors to capsules, into which the fibrils may condense. An alternative explanation for the capsule structure may be the polymerisation of a secondary type of fibrils upon the primary mesh (Fig. 5.4A), relegating the latter to a scaffolding function.
In either case, the fibril formation is not strictly synchronised between cells in the same culture. It rather appears to be a function of each cell’s individual age, because different stages appeared both on days 11 and 31 of incubation, as well as in between.

Figure 5.5: Fibrillar microstructures (arrow pairs) within capsule material of \textit{A. minutissimum} cells in xenic biofilm are revealed by mechanical stress (scale bars = 1 µm). Micrographs depict samples from an 11 days old culture. \textbf{A:} Tip of a partially encapsulated cell. Fibrillar substructures are continuous throughout the capsule material. \textbf{B:} Capsule material stretched between cells frays into fibrils.

To further elucidate whether or not fibrils and capsules might be related, we analysed mechanically stressed capsule areas (Fig. 5.5). Here, tension yielded an alignment of capsule microstructures, as well as fraying on the edges. Fibrillar structures resulting from these processes were similar in diameter to the frustule-covering fibrils.

There are two possible sources for the mechanical force. Firstly, motility of the cells relative to each other in the native biofilm. However, it has been reported for a related species in the order Achnanthales, that motility is lost upon production of EPS structures [Wang et al., 1997]. Secondly, mechanical force could be caused by the dehydration during SEM sample preparation. Both explanations lead to the question why mechanical force highlighted the fibrillar microstructure, while relaxed capsule areas (see previous figures) appeared unstructured. Micromanipulation experiments may be required to further investigate the properties of \textit{A. minutissimum} capsules and their fibrillar microstructures. Such experiments have been conducted by force-mode AFM on the mucilage layers of other diatoms [Higgins et al., 2003; Willis et al., 2013].

Based on this data, we suggest fibrils as a precursor candidate for capsules. Fibrils may condense into the capsule material as depicted in figure 5.4, for example by enzymatic cross-linking or transglycosylase activity. Fibrils may be disguised in relaxed capsule material because they are arranged in a disorderly fashion, but mechanical stress yielded a visible alignment.

**Capsule material does not contain silicon**

Silicon (Si) is a major component of diatom frustules, in which it is present as hydrated silicon dioxide. Capsules on the other hand may consist mostly of extracellular polymeric carbohydrates [Wustman et al., 1998; Windler et al., 2015]. In order to exclude the
posibility that the capsule material we observed might represent frustule deformations or extensions [Windler et al., 2014; Cantonati et al., 2014] it was screened for the presence of Si. Energy-dispersive X-ray (EDX) spectra were recorded from capsule areas with and without a cell body and thus a frustule below them (Fig. 5.6).

As expected, higher counts around 1.75 keV, which signifies Si [Guerra et al., 2013; Chandrasekaran et al., 2014], were obtained from capsule material, than from cell bodies. Background level Si counts likely result from the frustule edges close-by due to the “pear effect” [Arnould & Hild, 2007]. It explains, how excited electrons diffuse into the sample, so that a pear-shaped volume of ca. 0.5-1 µm diameter below the measurement point or area also emits detectable X-rays. The cell bodies in this figure are separated by approximately that distance. The stronger gold (Au) signal of the capsule material around 2.15 keV compared to the control areas probably resulted from the larger sputtered surface area within the measurement volume. Due to the absence of notable Si signals from *A. minutissimum* capsule material, we can exclude the possibility that it is some kind of frustule extension or deformation.

Furthermore, we can tentatively exclude chitin as a major component of the capsule material, because no notable nitrogen signals (N; 0.39 keV) were recorded from it. Chitin fibrils have been found to be secreted by diatoms into the surrounding water body [Gardner & Blackwell, 1971; Herth, 1979]. In contrast, the fibrillar meshes we describe here, tightly covered the frustule surfaces of individual *A. minutissimum* cells and therefore likely represent different EPS structures.

**Bacteria preferentially attach to encapsulated diatom cells**

It became apparent during the SEM observations, that diatom-attached bacteria cells occurred more often on capsules than on frustules. To substantiate this observation, bacteria cells were counted on both diatom cell surface types (Fig. 5.7).

Notably higher numbers of bacteria (ca. 25 times more on average) adhered to capsules than to frustules throughout the stationary phase (means: 11.41±8.23 and 0.46±0.82 respectively). The variance in the numbers of bacteria per diatom was larger (ca. 100 times) on encapsulated cells than on frustules, indicating that not all encapsulated *A. minutissimum* cells were equally strongly colonised by bacteria. Presumably, this is due to the population dynamics of the co-isolated bacteria within different areas of the xenic biofilms.

Bacteria as well as diatom cells with only fibrillar meshes on their frustule were found to individually retain attachment to the substrate. We deem it unlikely that attachment to each other could have been too weak to withstand the rinsing step. Instead, the bacteria preferentially adhered to capsules, while either being actively repelled from non-encapsulated *A. minutissimum* cells or only not especially drawn to their frustules.

It has been proposed that *A. minutissimum* capsules might be an asset in the mutualistic relationship of this diatom with its satellite bacteria [Windler et al., 2015]. Previous findings suggest a pattern of bacterial adherence to *A. minutissimum* cells in xenic biofilms that would support this hypothesis [Windler et al., 2012]: diatom cells were surrounded by a bacteria-free space, followed by a layer of densely aggregated bacteria cells. Although no CV stains were conducted in that study, the bacteria-free regions resemble the EPS structures reported since then as capsules.
Figure 5.7: Distribution of the number of bacteria cells adherent to diatom valve faces of different surface types (frustule or capsule) in xenic *A. minutissimum* biofilms. Bacteria were counted in SEM images, if they were in direct, visible contact with the valve face of either a frustule (*N* = 54) or a completely encapsulated diatom cell (*N* = 71; see figures 5.2B and 5.3A for illustration). Samples were taken from 11 to 31 days old cultures. Boxes represent the 1st and 3rd quartile. Black center lines represent medians. Diamond symbols represent means. Whiskers extend to 1.5-fold of the inter-quartile range (IQR). Black dots represent extreme values that lie outside the IQR.

In bacterial biofilms, nutrient distribution is predominantly determined by diffusion, sometimes along strong gradients within a biofilm [Stewart, 2003]. Similarly, variations of cellular nutrient distributions within freshwater diatom biofilms exist [Murdock et al., 2010]. Furthermore, it is possible that diatom capsules serve as a common nutrient pool to the satellite bacteria in a mutualistic relationship [Bruckner et al., 2008]. Therefore, competition between individual diatom cells for re-mineralising bacteria could occur. Nutrient-limited, but still photosynthetically active diatom cells may produce predominantly insoluble carbohydrates to foster close attachment of heterotrophic bacteria that re-mineralise EPS or secrete vitamins.

Our finding that bacteria attach preferentially to capsules strengthens the argument that capsules play a role in the inter-kingdom relationship of satellite bacteria and benthic diatoms. Whether this relationship is antagonistic, mutualistic or commensal in nature remains to be elucidated. Labelling experiments with isotopes or fluorophores may assist in the determination of carbohydrate fluxes from the diatom’s EPS structures to bacteria feeding on those.

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Chapter 6

Concluding remarks and perspectives

This thesis advances the state of the art in diatom-bacteria communication and biofilm research in mainly three ways (visualised in figure 6.1).

Firstly, a simple correlation technique for light and electron microscopy of biofilms was developed. It provided insights into both the hydrated biofilm, and the microstructure of unequivocally identifiable details within it. Thereby, frustule-attached fibrils were determined as a precursor candidate for the *A. minutissimum* capsule and potentially other diatom capsules. The discovery of these fibrils raises the question of their role in other stages of biofilm formation than the late encapsulation of cells.

Secondly, the multi-step extraction workflow for Bacteroidetes strain 32’s bioactivity worked in principle, but did not support the iterative improvement of the purification strategy well. Instead, a non-targeted, metabolomic approach may assist in elucidating not only the complex chemical interactions, but also the nutrient fluxes and availability discussed in chapters 2 and 5.

Thirdly, future screenings with the *A. minutissimum* bioassay were simplified by implementing robotisation options and a reproducible data processing workflow. These methodological improvements in particular support the replication of biofilm experiments to a higher degree of statistical confidence than previously achieved. This is exemplified by the datasets obtained by plate-reading (see figure 6, p. 80 and chapter 4, p. 47), and prepares the screening of knock-out mutants of Bacteroidetes S32 for biofilm-relevant phenotypes.

With these remarks in mind, several overarching findings, ideas and perspectives will be discussed in the following sections.
Figure 6.1: **Visual summary of this thesis.** Open research questions are question-marked. Biofilm formation of *A. minutissimum* is understood to be initiated by attachment of cells via mucous pads and the subsequent formation of stalks. Xenic cells attach constitutively, while axenic cells require inducer chemicals from Bacteroidetes S32. This induction is clearly mediated by hydrophobic compounds, whose identification will be supported by the now robotised biofilm formation assay. Cell division and the secretion of unstructured extracellular polymeric substances (EPS) contribute to the biofilm’s maturation, which is followed by capsule formation.
Application of correlative light-electron microscopy (CLEM) on biofilms

In this thesis, correlative light-electron microscopy was used to answer the question of how the A. minutissimum capsule was structured. As discussed in the previous chapter, the correlative approach was particularly useful for first visualising the presence or absence of EPS capsules around particular cells, and to secondly demonstrate other properties of those same cells, such as the bacterial attachment. While applying the simple technique of adding surface markers, the destruction of small biofilm sections was accepted, because it contained cells with and without capsules in an apparently random distribution (see Fig. 5.1, p. 65). Besides that simple, reproducible way of highlighting regions of interest (ROIs), a third goal was considered: reducing observational biases [Macleod et al., 2009]. This was achieved by applying markers almost blindly, after a quick microscopic screening of the stained samples for the expected overall biofilm density. The ROIs were subsequently selected under the light-microscope in relation to those markers, which remained visible during the preparation procedure of the samples for SEM. If the markers had been applied after selecting the ROIs, a larger bias may have been introduced. Selecting ROIs in denser areas of the biofilm for example, could have biased the counting of bacterial cells attached to the diatoms in an unforeseeable direction. This was due to the available data on the nature of A. minutissimum’s interaction with Bacteroidetes S32 allowing only hypothetical statements about possible mutualistic, commensalistic or parasitic interactions [Windler, 2014]. Without clear indicators for the direction and extent of observational biases, the author of this thesis recommends the simple, low-bias approach of adding ROI markers to homogeneous biofilm samples before the selection and in-depth observations of individual cell clusters for CLEM.

More advanced, biofilm-specific CLEM procedures could include the pre-inoculation marking of the sample carrier disks themselves [Mironov & Beznoussenko, 2009]. Uniquely asymmetric cuts could be made along a disk’s circumference, particularly when using plastic carrier material. Tri- and rectangular, as well as round, cuts for example should be distinguishable in both light and electron microscopy (LM and EM, respectively), regardless of the rotation of the disks. While the compatibility of cut disks with the fixation, dehydration and sputtering procedures would have to be validated, such cuts would mark the areas immediately surrounding them, and would provide a rudimentary coordinate system. In case the cuts are found to be incompatible, an alternative coordination technique could be adapted from the field of material science and engineering [Fejfar et al., 2015]: Indenting a biofilm surface at three asymmetrically placed points enables the triangulation of locations in the surrounding biofilm. In either coordinate system, more ROIs per disk could be mapped, thus providing more replicate data points and increased statistical power of the subsequent analyses.

Open biofilm research questions that could be addressed by CLEM are for example the origin and composition of the fibrillar structures which were found in varying lengths, degrees of meshing and differing 3D-structures (see figures 6.2 and 5.4, p. 68). Fluorescence- and immuno-labelling could be conducted against known extracellular proteins and/or carbohydrate structures. For example, fluorophore-conjugated lectins indicated the presence of α-N-acetyl-D-galactosamine in both the capsule material and other cell-wall-associated EPS [Bahulikar & Kroth, 2007]. These experiments could be repeated with the same lectins and A. minutissimum biofilms on the disk, but evaluated using high-resolution fluorescence microscopy. This may show the distribution of different carbohydrate types on frustules and capsules. Subsequently, either the same lectins, or anti-lectin antibodies, conjugated to tungsten or gold particles could be applied to the biofilms. Similar detection
techniques have been used for enteric bacteria, but with magnetic beads and coupled to
fluorescence-activated, flow-cytometric sorting [Porter et al., 1998; He et al., 2011] instead
of electron microscopy.

Figure 6.2: **Meshing and branching of A. minutissimum fibrils.** Which sugar com-
ponds underpin the surface-bound, protruding, meshing, or branching fibril structures?
This may be possible to elucidate by combining fluorescence staining of different carbohy-
drate species (for example via lectins [Bahulikar & Kroth, 2007] or modified oligosaccharide
[Späte et al., 2014]) with the correlative technique of depositing electron dense material at
the fluorescent sites [Grabenbauer, 2012]. Image was taken by Joachim Hentschel at 5 kV
EHT, 5.1 mm WD, 30 µm aperture and an in-lens signal detector. Scale bar represents
1 µm.

A more speculative approach to elucidating the composition of A. minutissimum’s
fibrils may be the injection of keto-, azido-, or alkene-labelled galactosamine analogues
into the diatom’s glycosylation pathways [Hang & Bertozzi, 2001; Dube et al., 2006; Späte
et al., 2014]. This process is called “metabolic oligosaccharide engineering” and exploits
the “permissivity” of enzymes in those pathways towards artificially modified substrates, while
avoiding the need to funnel bulky adducts (e.g fluorophores) through the glycosylation
pathways (see Campbell et al. [2007] for a review). A potential target pathway may be
the O-glycosylation of serine and threonine with N-acetylgalactosamine (GalNAc), which
is also known as the mucin-type glycosylation and is very common in animals [Dube et
al., 2006]. Mucin-like domains have also been identified in P. tricornutum as a novel
class of soluble, extracellular diatom proteins which may be involved in biofilm formation
[Buhmann, 2015]. Potentially, fully soluble, as well as extracellularly attached variants
of this protein class exist. Therefore, the possible localisation of mucin-like domains in
A. minutissimum fibrils could be investigated by metabolic oligosaccharide engineering of
modified GalNAc. Unfortunately, the uptake of such modified sugars was found to be
low in the diatom model P. tricornutum [personal communication, Lili Chu & Jochen
Buck]. The artificial introduction of a promiscuous GalNAc transporter may be necessary
to ensure the uptake of modified sugars. Such an introduction was achieved by Zaslavskaia
et al. [2001] for the glucose transporter GluT in P. tricornutum, yielding the so called
“trophic conversion” of the diatom. Bypassing the complexity of this technique and of
the genome engineering (briefly discussed in chapter 3) in the eukaryotic diatoms, feeding
experiments with modified sugars could instead be conducted with A. minutissimum/S32
co-cultures, or the xenic diatom. Here, the bacterium’s contribution to the EPS structures and the biofilm matrix could be investigated.

Regardless of which fluorophore delivery method is chosen, the correlation to EM may be achieved by utilising a technique reviewed by Grabenbauer [2012] for GFP-tagged proteins. In summary, photo-bleaching and -oxidation of the fluorophores can be used to locally deposit diaminobenzidine polymers, which can be stained by osmium tetroxide for EM. In future biofilm research, such deposition techniques of electron dense material could be applied to modified proteoglycans on cell surfaces. In addition to the analytic identification of different carbohydrate species in the EPS of *A. minutissimum* [Bahulikar & Kroth, 2008], they could thus also be localised more precisely than by fluorescence microscopy alone.

Another research question concerns the distribution of elements and chemicals in the *A. minutissimum* frustules and capsules. The energy-dispersive X-ray (EDX) spectra that were measured for figure 5.6 (p. 71) could be supplemented with a different correlative SFM and ToF-SIMS measurements (scanning force microscopy and time-of-flight secondary ion mass spectrometry, respectively; Bernard et al. [2014]; Sometimes also called “nanochemistry”). The reported implementation of SFM and ToF-SIMS in the same vacuum chamber supports the correlation of the three-dimensional surface topography of a sample with the localised identification of elements and chemicals from that surface. By this method, organic structures composed of proteins [Polemis et al., 2013], lipids [Anderton et al., 2011] and sugars [Wehbe & Houssiau, 2010] were characterised. Unfortunately the lateral resolutions in those studies was too low for the few dozen nanometers thick fibrils discovered in this thesis. Because proteins and sugars are expected to be the principal components of the EPS in fibrils and capsules of *A. minutissimum*, SFM/ToF-SIMS may be an interesting option once devices with higher resolution become available.

**Future investigation of the bioactivity of Bacteroidetes strain 32**

Bacteroidetes S32 was isolated from phototrophic biofilms of Lake Constance by Bruckner et al. [2008] and initially placed into the *Dyadobacter* genus. The present thesis confirms this taxonomic placement on a larger BLAST dataset, as well as by the more reliable bioinformatic method on the RDP dataset, see section 3 (p. 37). Nevertheless, the exact species of S32 remains to be elucidated. That endeavour may be assisted by a complete genome sequencing, which may enabling the search for genes that are relevant for the production and secretion of secondary metabolites. Moreover, phenotypic and metabolic characterisations may help identify S32 as a species. Bochner et al. [2001] reported on a micro-array system to simultaneously test nutrient metabolisms and many other phenotypes in a bacterial strain. Such an approach may help determine S32’s essential nutrients, as well as other metabolic properties and chemical sensitivities. The findings could inform the preparation of high-yield growth media, with only the minimal set of essential ingredients. Possibly, the question of which LB ingredients effected the notably denser S32 growth than in the recommended *Dyadobacter* media 1 and 830 (see Fig. 3.1, p. 37) could be answered. Moreover, media could additionally be supplemented with labelled compounds, in order to elucidate whether S32’s biofilm inducers are synthesised from complex precursors, or *de novo* from basic nutrients. In the either case, S32’s chemical cues might be obtained at higher purities in the minimal supernatant, than in LB, which would support future purification efforts.
In case the bioassay-guided approach is continued, the possibility of synergistic action of different chemicals should be considered. Despite high yields and starting purities, a bioactivity may disappear during the chromatographic separation of the bioactive chemicals. In that case, their successful identification would require many fraction combinations to be tested. The number of required tests can be reduced logically by implementing a subtractive-combinatorial purification and assay strategy [Byers, 1992]. Therein, separated fractions are mixed, assayed and, if bioactive, reproduced with fewer fractions in a binary search pattern. Bioactive fractions are identified via the loss of bioactivity of those mixtures that do not include those fractions. Regardless of the number of samples to be tested in this approach, the simplified and robotised bioassay protocol presented in chapter 4, p. 47) may assist in any future bioassay-guided purification of the infochemicals in the *A. minutissimum* and S32 bioassay.

Also, it remains to be investigated whether the transposon mutants of S32 that lacked a growth-enhancing phenotype for the diatom *Fragilaria brevistriata* [Windler, 2014] were affected in types of secretion systems relevant to non-protein compounds, which the resilience of S32 bioactivity to harsh conditions suggests. Such studies may help elucidate whether the enrichment of biofilm-inducing substances from S32 in its liquid culture supernatant is the result of an active process or simply cell lysis over time. Mutants with biofilm-relevant phenotypes following secretion system knock-outs have been investigated in the phylum Bacteroidetes, albeit with a human pathogen [Tomek et al., 2014]. They found denser cell aggregation in biofilms of mutants which lacked the type IX secretion system T9SS, compared to wild types with intact T9SS. That secretion system normally translocates glycosylated proteins with certain conserved C-terminal domains through the bacterium’s outer membrane. In a cyanobacterial context, it was demonstrated that an impairment in protein secretion and pilus assembly via type II and IV systems relieved the self-inhibition of biofilm formation [Schatz et al., 2013]. These cyanobacteria can thus regulate their lifestyles between planktonic and sessile. Such regulators may be infochemicals to other organisms (as for example suggested by Amin et al. [2012]) which induce lifestyle adaptations in response to the chemical stimuli of other organisms in the same ecological niche.

**Non-targeted approaches to the analysis of Bacteroidetes strain 32 supernatant**

As discussed in chapter 3 (p. 31), the targeted, bioassay-guided approach to the fractionation of Bacteroidetes S32’s bioactivity was suboptimal. For future work on the discovery of infochemicals in the *A. minutissimum*/S32 model system, a non-targeted, metabolite analysis of only solid-phase extracted supernatant should be attempted. One such analysis method is the pyrolysis field ionisation mass spectrometry (Py-FIMS). Pyrolysis is defined as the chemical degradation of a sample, driven solely by thermal energy in an inert environment [Ericsson & Lattimer, 1989]. Field ionisation applies strong electric fields to ionise samples in a soft, fragmentation-avoiding manner [Lehmann & Schulten, 1976]. Thus, the mass spectra of field-ionised samples can be assumed to represent the sampled molecules more accurately than fragment-forming techniques, such as electron spray ionisation. This combination of temperature-controlled release of chemicals from a sample and their non-fragmenting ionisation makes Py-FIMS particularly useful for analysing the composition of unknown substances [Leinweber et al., 2009]. In order to test the applicability of this technique, and in an attempt to analyse extracts of Bacteroidetes strain 32
supernatant with a non-targeted method in parallel to the bioassay-guided fractionation, samples with and without biofilm-inducing activity were sent for Py-FIMS analysis.

Figure 6.3: Py-FIMS spectra of negative control (LB) and biofilm-inducing supernatant (S32). Inlays display aggregated evaporation intensities during the step-wise temperature increase during the pyrolysis. In both spectra, a peak at 390.264±0.025 m/z is present and roughly correlates in its intensity with the strength of biofilm induction of the sample.

The Py-FIMS analysis of sterile LB and S32’s spent supernatant (Fig. 6.3) revealed that the LB contained more substances with evaporation temperatures of 200-400°C than the supernatant. Conversely, the latter contained more substances with evaporation temperatures lower than 200°C, indicating the conversion of nutrients to more volatile metabolites. This lower temperature range should be considered for future Py-FIMS analyses of S32’s biofilm-inducing secretions.

In order to continue the metabolomics approach, Py-FIMS could also be conducted with S32 supernatant samples from less complex media. The complex LB medium was used for S32 cultivation here, because maximum yields of cell mass and thus bacterial secretions in the supernatant were prioritised over the purity of the starting supernatant. However, sensitivity of Py-FIMS has been demonstrated in the context of tracking diurnal rhizodeposition profiles of maize [Kuzyakov et al., 2003] and in rhizodeposit comparisons of genetically modified potato crops [Melnitchouck et al., 2006]. It is therefore reasonable to assume that Py-FIMS is sensitive enough for the detection of bacterial compounds in medium supernatants without cell density maximisation.

Another non-targeted, metabolomics approach could adapt the two-chamber co-culturing system of Paul et al. [2012] to our A. minutissimum and S32 model system. The system is composed of two glass chambers between which a membrane with cell- and potentially protein-excluding pore sizes is placed. The axenic diatom could be cultivated in one of the chambers first, with regular sampling of secreted, diffused compounds from the cell-free second chamber. By analytical methods such as gas chromatography and mass spectrometry, a baseline of secreted metabolites of axenic A. minutissimum could be established. As previously shown, soluble sugars will likely be among these secreted compounds (Fig. 2.6, p. 26) and may support the later inoculation of heterotrophic S32. The bacterium could be inoculated in either (a) the first chamber, so that cell-free sampling can be continued from the second, or in (b) that second chamber, so that direct contact between diatom and bacteria cells can be excluded, or in (c) a third chamber which ensures both these properties and can be crafted by a glass blower. In either case,
repeated analyses of the co-culture’s diffused supernatant composition over time may assist in the identification of secreted, soluble substances from either organism.

**Application of the *A. minutissimum* biofilm assay to the screening of pure chemicals**

So far, the bioassay was applied in the assessment of biofilm-inducing effects of supernatants or extracts from bacterial liquid cultures. This combined the problem of laborious sample preparation with the inherent incubation time of at least one week for the diatom cells. As concluded in chapter 2, the assay may also be applicable to the screening of pure, commercially available chemicals. Circumventing the laborious sample preparation, the following chemicals were assayed for biofilm-inducing or -inhibiting effects:

- The plasticiser di-n-octyl phthalat (DNOP or DEHP, CAS: 117-84-0), because it was enriched in biofilm-inducing extract fractions (390.264±0.025 m/z peak in Fig. 6.3, p. 79) and had previously been detected in water samples known to have been in contact with soft plastic tubing [Kai-Uwe Eckhardt, Uni Rostock, personal communication], which was a potential source in this thesis as well.
- L-homoserine lactone (HSL, CAS: 2185-02-6), because it is a commercially available building block of the bacterial quorum-sensing chemical acyl homoserine lactones (AHLs). These have been suggested as potential infochemicals to diatoms, due to their hydrophobic, membrane-permeating nature [Amin et al., 2012].
- Indol-3-acetic acid (IAA, CAS: 87-51-4), because it can be extracted from solid C18 phases with highly methanolic eluents also [Hou et al., 2008], and because it was recently identified as a promoter of diatom cell division in marine consortia of *Pseudo-nitzschia multiseries* and a *Sulfitobacter* strain [Amin et al., 2015]. We tested the same nanomolar concentration range that was found to significantly increase *P. multiseries* growth.

As figure 6.4 illustrates, neither the basic compound of bacterial quorum-sensing chemicals (HSL), nor the recently discovered cell division promoter (IAA), nor the plasticiser (DNOP) are biofilm-inducing infochemicals to *A. minutissimum* in those concentration ranges. The DNOP and IAA data fluctuate slightly, but only around the on average evenly linear trend. That trend is most clearly pronounced in the HSL data which was gathered in the highest number of replicates. Absorbances of solvent controls (0 M) were in accordance with the respective linear trends. In all cases, the biofilm-positive, xenic controls showed an at least 2-fold stain intensity. DNOP and IAA were also tested on co-cultures and did not result in biofilm inhibition (data not shown). The generally higher absorbance in the DNOP-experiment resulted from the application of the manual bioassay protocol (ch. 2, p. 16), while HSL and IAA data was obtained by the semi-automated protocol (ch. 4, p. 47).

Plasticisers have been recognised as contaminants in aquatic environments generally (see review by Cole et al. [2011] and references therein). Phthalate esters in particular are ubiquitously found in environmental water samples [Wang et al., 2011; Hope et al., 2012; Polyakova et al., 2014]. It is unclear, why a phthalate ester would accumulate alongside S32 growing in LB, because such compounds are on the contrary understood to be degradable by bacteria [Keyser et al., 1976; Stales et al., 1997; Cartwright et al., 2000]. Apart from coincidental, its correlation to the bioactivity might be a result of
Figure 6.4: Biofilm induction of *A. minutissimum* by a selection of chemicals. CV absorbance of adherent cells in cultures treated with DNOP was determined with a photometer after EtOH extraction, while HSL and IAA data was obtained by plate-reading (*N* = 3 (DNOP), 6 (HSL) and 2-4 (IAA) per concentration). Shaded area represents confidence interval of 95% around linear regression according to Wilkinson & Rogers [1973].

correlation with the actual infochemical(s) and other hydrophobic substances from S32’s spent supernatant. Seemingly no specific DNOP tests with diatom biofilms have been published so far. Together with larger phthalates however, DNOP is a known inhibitor of *Staphylococcus aureus* and *Escherichia coli* attachment to PVC membranes [Chapman et al., 2010]. Also, Sakata et al. [2011] suggested DNOP to be a component of an algicidal chromatographic fraction of a marine *Pseudomonas* strain, but the pure DNOP did not possess bioactivity itself in further tests. Instead, they identified 2,3-indolinedione as the algicidal compound (also called isatin1; CAS: 91-56-5. This is in contrast to the apparently beneficial effect of the other indole derivative IAA found by Amin et al. [2015]. There are also artificially produced indole derivatives with the opposite effects on diatoms. Yang et al. [2015] found that several halogenated indoles inhibited growth of two marine biofouling diatoms better than copper sulphate, another commonly used anti-fouling agent. Moreover, 6-chloroindole was found to trigger Ca$^{2+}$ efflux from *Cylindrotheca* sp. [Yang et al., 2014]. Apparently, the indole moiety is an infochemical structure to several diatom species, and elicits a range of different responses. It would therefore be interesting to (a) screen such indole derivatives in our *A. minutissimum* biofilm assay, and to (b) optimise analytical procedures for the detection of indoles in the supernatant of Bacteroidetes S32.

Another biofilm-relevant chemical is 2E,4E-decadienal. It is a diatom-derived polyunsaturated aldehyde that inhibits biofilm formation of the benthic diatom *Fistulifera saprophila* [Leflaive & Ten-Hage, 2010]. The growth inhibiting concentration of 52.5 µM, the adhesion inhibition above 6.6 µM, as well as the reduced cell motility at 16.5 µM are all roughly 100 to 500 times higher than the concentrations of bacteria-derived IAA that promoted cell division in *P. multiseries* [Amin et al., 2015]. This illustrates the diversity

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of effect types, sources and concentration ranges of diatom infochemicals. While IAA was found to not induce biofilm formation in axenic *A. minutissimum*, 2E,4E-decadienal could be tested in the future with substantially higher replicate numbers than previously, thanks to the upscaling methods presented in this thesis. Hypothetically, 2E,4E-decadienal would not induce biofilms in the axenic diatom either, but it may suppress biofilm formation by xenic *A. minutissimum* and co-cultures of the axenic diatom with Bacteroidetes S32.

**Further up-scaling potential of the *A. minutissimum* biofilm assay**

Several aspects of the initially manually conducted bioassay were simplified and sped up by applying automation and data pipelining techniques (see chapter 4, p. 47). Thus, the *A. minutissimum* bioassay may be applicable for medium-throughput screenings of biofilm-relevant bacterial mutants or chemical substances. The assay’s long incubation time would not hinder the screening of such samples as much, as it did the supernatant purification discussed above. Particularly for medium-throughput use-cases, the plate read-out may be simplified further, such that biofilm quantification is completely omitted in favour of a visual, qualitative assessment. The presence or absence of biofilms in this assay could be detected by eye, similar to bioassays with visually distinct inhibition zones or microbial colonies (see section 5.4.1 of Windler [2014]) or a “BioFilm Ring” test based on magnetic microbeads [Chavant et al., 2007; Bernardi & Bara, 2011]. In the latter test, the beads are inoculated and dispersed together with the cultures and (unless biofilms are formed during the cultivation), a visible aggregation of the beads can later be induced by magnetic fields. However, this does not appear to be necessary in the *A. minutissimum* bioassay, because laboratory experience showed, that the cells themselves can be visibly aggregated simply by gentle shaking of the 48-well plates.

A higher throughput of samples could also be achieved by further-reaching robotisation of the inoculation of *A. minutissimum* and the application of treatments. Although this would be in contrast to the simplification discussed above, it may be possible with the Tecan Freedom EVO modular, robotised worktable, as demonstrated by Tillich et al. [2014] for cyanobacteria. In those semi-sterile conditions, it would be important to control S32 and *A. minutissimum* cultures for biofilm-modulating effects of possible contaminations. While S32’s specific [Bruckner et al., 2008] and constitutive (see Fig. 2.4, p. 23) infochemical secretion seems unlikely to be disrupted, the axenic control cultures may be. They may therefore require antibiotic shielding, or should alternatively be controlled for unintended biofilm formation by intentional exposure to the surrounding air. Alternatively, the whole robotic set-up could be encapsulated in a sterile environment as suggested by Tillich et al. [2014]. Besides microalgae cultivation, they also reported on a variety of add-on measurements, of which the MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass-spectroscopy) is particularly relevant to the search for bacterial infochemicals. The S32 knockout strains produced by Windler et al. [2014] could be cultivated in parallel, and their supernatants could be screened for metabolomic differences. Mass signals that are present in S32 wild-type, but absent from mutants that do not induce axenic *A. minutissimum* biofilms would be infochemical candidates.

Because that level of robotisation would require the switch from 48- to 96-well plates [personal communication, Silke Müller], the read-out method may have to be adjusted. Up to now, liquids were manually pipetted out of the wells before the absorbance read-out, but the wells were not additionally dried. The well area covered by the meniscus of residual liquid was small, so the measurement’s light path could have been diffracted
slightly. In smaller wells, the area under a meniscus of residual liquid would be relatively larger around the well edge, so that a smaller middle area may be available for undiffracted measurements. The spread of measurement points over that well middle area would also be smaller, so the statistical analysis would be less representative of the sample. A second effect might also complicate the switch to 96-well plates: Rasmussen & Østgaard [2001] observed the inverse case of meniscus-formation by culture gels in multi-well plates, which made it impossible to map cell distribution. In our bioassay, the distribution of cells on the well bottom affects the photometric read-out results. This distribution may change due to almost unavoidable shaking during plate transport: esp. non-adherent cells (e.g. negative, axenic controls) may aggregate in the well middle. In 96-well plates, the possibly larger meniscus might restrict the area of undisturbed measurement exactly into this aggregation center. Previously, alternative methods for distinguishing wells with non-adherent, axenic A. minutissimum from wells with induced biofilms, were discussed. In addition to those, a faster removal of non-adherent cells, standardised shaking to reproducibly resuspend non-adherent cells, and/or a more thorough, robotised liquid removal may be solutions to the problem of meniscus-restricted measurement area. Regardless of the direction that further methodological changes to the A. minutissimum bioassay may take, the plate-read datasets shown in figure 6 (p. 80) and chapter 4 (p. 47) already exemplify the advantage of semi-automated measuring and processing of experiments with many replicates. This will support a qualitative identification of the biofilm-inducing chemical(s) of Bacteroidetes S32, as well as the quantification of their bioactive concentration ranges.
CHAPTER 6. CONCLUDING REMARKS AND PERSPECTIVES
Supplement

Please note: The printed version of this thesis includes a CD with the referenced supplemental files. The .pdf version includes these files as attachments. PDF viewer permitting, they can be opened or saved directly at “available here”-notes. Alternatively, referenced files will also be available at github.com/katrinleinweber/PhD-thesis.

Chapter 2: Biofilm and capsule formation of the diatom *Achnanthidium minutissimum* are affected by a bacterium

Colorimetric analysis of other carbohydrate standards

![Figure 6.5: Comparison of glucose and glucuronic acid standard curves in Phenol-sulfuric acid assay (N = 1). The largest deviation from both the mix and glucose was found for glucuronic acid (dashes and dotted line). Its linear regression slope is 81 and 88% more shallow respectively, which indicates that this carbohydrate compound either reacts incompletely or yields a products with low absorbance around 488 nm. In either case, it can only contribute little to the EPS concentrations we report in this.](image-url)
Figure 6.6: **Standard curves of several carbohydrates prevalent in *A. minutissimum* (N = 1).** In addition to the glucose-based calibration of the phenol-sulfuric acid assay that was used in this study, six most abundant compounds found in *A. minutissimum* previously by Bahulikar & Kroth [2008] were tested. Dilution series of the standards in natural mixture, as well as individually, yielded the calibration data. Glucose (diamonds and dashed line) exhibits the most pronounced colorimetric reaction. Other sugars have up to 61% more shallow regression slopes. As expected, the natural mix (x with solid line) yielded an average linear regression slope, compared to which the one of glucose is 34% steeper.
Growth of Bacteroidetes strain 32 in diatom full medium

![Graph showing OD for LB and BM media over time](image)

Figure 6.7: Bacteroidetes strain 32 was not able to grow in diatom full medium (BM) without the diatom *A. minutissimum* or the glucose supplement. The bacterium was cultivated in BM medium (*N* = 2) and in diluted LB (*N* = 1). Bacteroidetes strain 32 was inoculated in the diatom medium BM and in diluted LB (50% v/v). Therefore, the bacterium was scrapped from an agar plate containing diluted LB, resuspended in 1 mL BM and 10 µl were added to 5 mL of the respective medium in glass reaction tubes. Cultures were incubated at 22°C and 112 rpm. OD$_{600}$ was measured with an M107 spectrophotometer (Camspec Ltd, Cambridge, UK).

Quantification of soluble and bound EPS

In a preliminary test we observed the extractability of the capsular material. We therefore observed the cell pellets after treatment with warm water (WW), hot water (HW), hot bicarbonate (HB) and hot alkali (HA) by light microscopy. Extraction of carbohydrates was performed as described in section *Quantification of soluble and bound EPS* (p. 18) with the only difference that the fraction containing the soluble polymers was filtered using a 0.2 µm filter previous to evaporation. To highlight bound carbohydrates, the pellets were stained with alcian blue.

Figure 6.8 (following page): Quantification of carbohydrates and microscopic images of the xenic and axenic *A. minutissimum* cells in different extracts. Xenic *A. minutissimum* cells exhibited capsules even after treatment with warm water and defatting (A and B). Hot water treatment results in a loosening of the capsule structure (C). The capsules were completely dissolved after treatment with hot bicarbonate (D). Treatment with hot alkali solution dissolved all cell structures including the frustules (E). The carbohydrate profile of the xenic culture correlates well with the dissolution of the capsule material as it shows high carbohydrate contents in the HW and HB fractions.
Co-cultivation of *A. minutissimum* with different bacterial isolates

Bacteria isolates B-1, B-2, B-4, B-5, B-6, B-7, B-8 and B-10 were isolated from phototrophic, epilithic biofilms from the same sampling site as *A. minutissimum* and Bacteroidetes strain 32 in April 2011. The biofilm was scraped from stone surfaces, diluted in BM and roughly vortexed for 10 min before the suspension was plated in gradual 1:10 dilutions on agar plates containing diluted LB medium (50% (v/v)). Single colonies were picked and isolated by repeated smear. The bacterial isolates were cultivated as described for strain 32. For co-cultivation, 1 mL BM was inoculated with $2.8 \cdot 10^4$ diatom cells/ml and 5 µl of the bacterial cell suspension (OD$_{600}$ 0.1). The co-cultures and negative controls (axenic diatom and bacteria, respectively) were performed in triplicates, the positive control (co-culture of *A. minutissimum* with strain 32) in duplicates. Biofilm was stained with CV and the absorption of the extracted dye was measured at 580 nm.

![Graph](image)

Figure 6.9: Absorption of crystal violet (CV) extracted from biofilms of co-cultures of *A. minutissimum* with different bacterial isolates. Three co-cultures produce a clearly stronger biofilm than the axenic diatom culture, co-cultures with B-1, B-5 and B-6). Only the co-cultures with B-1 and B-5 achieved a biofilm quantity comparable to the co-culture with strain 32. Co-cultures with B-4, B-7 or B-8 showed comparable or even less CV absorptions as the axenic diatom culture. Pure bacteria cultures in BM resulted in low or even no biofilm formation as they presumably did not grow in the used medium without diatom or additional carbon sources. Capsule formation was only visible in the co-culture with strain 32.
Colorimetric analysis of defatting fractions during quantification of bound EPS

In order to check for leakage of intracellular carbohydrates into the defatting fractions during the extraction of bound EPS, the procedure described in section *Quantification of soluble and bound EPS* (p. 18) was repeated with one stationary axenic and xenic culture each and the EtOH fractions were collected. These defatting fractions were evaporated and taken up in 1 mL ultrapure water. These samples were analysed in the phenol-sulfuric acid assay as described in this study using the standard curve of the mixture described above (Fig. 6.6)

![Figure 6.10: Quantification of carbohydrate release from cells during defatting steps with EtOH ($N = 1$). Both cultures show similarly high release of presumably intracellular carbohydrates in the first defatting step with EtOH. No carbohydrates could be detected in the following steps which indicates that the pellets did not contain intracellular carbohydrates any more. Furthermore, chl was extracted from the defatted pellet as described in section *Determination of chlorophyll (chl) concentrations* (p. 19).](image-url)
Figure 6.11: **Quantification of chl residue in defatted pellet (N = 1).** After defatting of the axenic and xenic pellet, only 4.8 and 1.4% respectively of the cultures’ initial chl concentrations were found. A pale, greenish-yellow colouration of the pellet indicated the possibility of small quantities of chl remaining protected by well-developed capsules. However, given these data and the observed green colouration in the first few EtOH fractions, we conclude that the vast majority of chl was extracted by the defatting steps. This in turn means that defatting efficiently breaks open even encapsulated, xenic *A. minutissimum* cells.
Chapter 3: A bioassay-guided fractionation of bacterial infochemicals that induce biofilm formation by *Achnanthidium minutissimum*.

The following good quality 16S ribosomal DNA sequence parts (forward and reverse, respectively\(^2\)) of Bacteroidetes strain 32 were used for the taxonomic placement as described in section Sequencing of the 16S ribosomal RNA gene of Bacteroidetes strain 32 (p. 33).

1 CTGCAAGCGG CGCGACTAGT GATTAGGT TGATCTGCC TGAGGATGA CGCTACGGC  
61 AGGTTCCTAA CAGTCAGGCG AGGGGGCGAG AAGTGTACCG GTCGTCAAGG TGCCGCAAGC  
121 GTACTGACCC TACCTATCTA TGCCGGGTAA CGCCGATGAA TACCGCATAA  
181 CACAGGGTCC CGCGCTTGGT ACTATTGTTT AAAGATTTTA CGTGCTGAGA TGCCGATGCG  
241 TTGATAGGAG TAGTTGTATA ATGTAACGCC TATAAGCTAG AGGAGTGCCT CTCCGATGTA  
301 AGAGGGTCTGAGG GCCGACTGAG TACCGGGCAG ACTCTAGAG GAGGCAGCG  
361 TAGGGGATAT TAGGGCTGCTG ATGCAAGGCT GCCAGCAAGC CGAGATGAGG  
421 CCTCCAGGTT GTCAGACGCC TTTTATTGGA AAGAAGGAGG AGGAGTGCCT CTCCGATGTA  
481 CGGCTCAGAG TGAATAAGAG CCGCTACTAA CGTGGCAGAC AGCGGGGCTG ATACGGAGGG  
541 TCGAGGCTTT TCCGGTACTT ATGGGTTTTA AAGGTTGCTG AGGGTGTCTG TAAATGCTAG  
601 GTGAAATTAC AGGCCGCTCAA CGTTTGTGGC GCCATTGATA CTGACAGCTG TGAACAACTG  
661 GAGGGCTGCG GGAATGCGAG GTGATGGCCT GAAATCTGTA GATATCTAGC AGAACACCGA  
721 TCGGCAAGGG AGGGGTGCTAC CTGGATATTG ACATGAGGAC AGCACAAGGAGGGGCACA  
781 CAGGATTAGA TACCTCTGTA TGGCATGCC TAAACGATGA GGACTCCTG CTGGCCTGCT  
841 AAGGAGGCAG GCCTGCGTTT AAGGGAACTG CTCAGCACCT CGACAGATGG GCCGCAAGCG  
901 TAAACTCGA AAGAAATCGG GGGGTGCCGG ACAAGGCTTT GACCATGGG TTTAACCTGA  
961 TGGATACGCG GGAACCTTAC CTGGCTCAGA TCACTACTAG CGGATTACGC ATGCGTGTTT  
1021 CCAGCAATCGT CTGGTGCCAG GCTGGCTGCAG CTGCTGCTGT AGCTGCTGT

2 Complete sequences and RDP classification results are in Supplemental File S32-16S-rdp-classified-hierarchy.txt (available here)
The following 16S ribosomal DNA consensus sequence\(^3\) of Bacteroidetes strain 32 was used for the BLASTN analysis which is summarised in table 6.1.

\[
\begin{align*}
1 & \text{ GCCCGGGAA ACcCGGATTA ATACCCGATA ACACCGGGGT CCCGATGGG TACTATTGTTATCACTCCAGCA GAGGGAGCT GAGAGTTGCA TCCCCACAC GGGCAGTGG } \\
61 & \text{ TAAAGATTAA CGGCTGATTG AGTGCCATCC GTGCGATTAG CTAGTGTATG TAGTGAACCG } \\
121 & \text{ CTTACCAAGG CTACGATCGA TAGGGGAGCT GAGAGTTGCA TCCCCACAC GGGCAGTGG } \\
181 & \text{ ATACGGGGCC GACTCCTACG GGAGCGAGCA GTAGGGAATA TTGGGCAATG GATGCAAGTC } \\
241 & \text{ TGACCCAGGC ATGCGCGGCT GGGATGAAG GCCCTGAGG TGTTAAACCG TTTTATTGCG } \\
301 & \text{ GGAAGAAGAG CACGGATCCG TCTTGTGTG ATGGACCGCA ATGAAATAGC ACCGCTAAC } \\
361 & \text{ TCCGTGCGAG CAGCGCAGGT AATACGGAGG GTGCGAGCCT GTCCGGATT TATTGGTTTATC } \\
421 & \text{ AAGGGTGCCG TAGTTGGCCT GTTAAGTCA GAGGGCCCTC ACGGCTGGG } \\
481 & \text{ TGCCATGTG ACTGACAAGC TTGAAACAG TGGAGGCTTC CGGAATGGAT GGTGACGCG } \\
541 & \text{ TGAATATGC ATGATACATC CAGAACACCG ATGGCGAGG AAGTGGTCACT CGTTGATTTT } \\
601 & \text{ GACACTGAGG CACGAAAGCA TGGGGAGCAA AAGGATTAGG ATACCCCTAG ATGCCATGCC } \\
661 & \text{ GTAAACGATG ACGACTGCT GGGCGCTTGT CAAAGGTCAAG CGTCTTGAAG AACCCTGTA } \\
721 & \text{ GTCCTCCACC TGGGGAGTAC GCGCGCAAGC GTGAAACTCA AAGGAATTAC CGGGGTCCG } \\
781 & \text{ CACAAGCGGT GGGAGATGTG GTTAAATCTC ATGATACCCG AGGAACCTTA CCTGGGCTAA } \\
841 & \text{ ATCACCACAG GAATCTTAT CAAAATTGGTG ATCCGACATG GCGTTGTTTT AAGTGGTCTGC } \\
901 & \text{ ATGGCTGTGC TCAGCTGTGC TCGTGAATG TGTTGAAGAC TCCGCCAACG AGCCGACCCC } \\
961 & \text{ CTATGGTTTAC TGGCGCACG GTAATGGGCG GGGACTCTAA TCAGCTGACC TGTGCAACCA } \\
1021 & \text{ AGAAGAGGAAGG GAGGGGACGA CGTCAAGTCA TCATTGGGCA TTTACGCTCA GGGGCAAAC } \\
1081 & \text{ AACGCTCTTA CAaTGCGCGG GTACAAGGG TTAGCTACC TCCCACCGATG AGAATGCCAA } \\
1141 & \text{ TCCCCAAAG CGGTtTCGCC AGttTCGCCaT TGAAATCTG CACCTCGACT CTTATGGGAAG } \\
1201 & \text{ aCTGGAATAC CTTAgATAA TCCCCCCCACC CCTATGGGg gCaGTTGaaAA Aaa }
\end{align*}
\]

\(^3\) S32-16S-consensus.fas (available here)
Table 6.1: **BLASTN hits of 16S ribosomal DNA consensus sequence from Bacteroidetes strain 32.** Abbreviated genus name *D.* stands for *Dyadobacter,* and *P.* for *Persicitalea.* Query and hits are partial sequences with lengths around 1.25 kb, E values of 0.0. Query covers of 97%. Max and Total scores were equal, and are thus summarily listed as Score here. Row with dashes (–) indicates the gap of 480 score points and 7 percentage points in identity that separate *Dyadobacter* hits from the next closest genus. Repeating the BLASTN analysis with the 16S sequences of these hits again yielded *Dyadobacter* clusters with the same ranges of query covers and sequence identities, as well as a >6%-point gap to the next closest genus.

<table>
<thead>
<tr>
<th>Species (strain)</th>
<th>Score</th>
<th>Identity</th>
<th>Accession (NR_)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. koreensis</em> (NBRC 101116)</td>
<td>1873</td>
<td>95%</td>
<td>113977.1</td>
</tr>
<tr>
<td><em>D. koreensis</em> (KCTC 12537)</td>
<td>1873</td>
<td>95%</td>
<td>044041.1</td>
</tr>
<tr>
<td><em>D. psychrophilus</em> (BZ26)</td>
<td>1868</td>
<td>95%</td>
<td>117212.1</td>
</tr>
<tr>
<td><em>D. ginsengisoli</em> (Gsoil 04)</td>
<td>1838</td>
<td>94%</td>
<td>041372.1</td>
</tr>
<tr>
<td><em>D. hamtensis</em> (HHS 11)</td>
<td>1838</td>
<td>94%</td>
<td>042226.1</td>
</tr>
<tr>
<td><em>D. jejuensis</em> (AMIR11)</td>
<td>1825</td>
<td>94%</td>
<td>109488.1</td>
</tr>
<tr>
<td><em>D. fermentans</em> (NS114)</td>
<td>1794</td>
<td>94%</td>
<td>027533.1</td>
</tr>
<tr>
<td><em>D. alkalitolerans</em> (12116)</td>
<td>1790</td>
<td>93%</td>
<td>044476.1</td>
</tr>
<tr>
<td><em>D. soli</em> (MJ20)</td>
<td>1788</td>
<td>93%</td>
<td>117263.1</td>
</tr>
<tr>
<td><em>D. tibetensis</em> (Y620-1)</td>
<td>1777</td>
<td>94%</td>
<td>109648.1</td>
</tr>
<tr>
<td><em>D. arcticus</em> (R-S7-29)</td>
<td>1757</td>
<td>93%</td>
<td>109479.1</td>
</tr>
<tr>
<td><em>D. crusticola</em> (CP183-8)</td>
<td>1744</td>
<td>93%</td>
<td>042335.1</td>
</tr>
<tr>
<td><em>D. beijingensis</em> (A54)</td>
<td>1705</td>
<td>92%</td>
<td>043725.1</td>
</tr>
<tr>
<td><em>P. jodogahamensis</em> (NBRC 103568)</td>
<td>1225</td>
<td>85%</td>
<td>114246.1</td>
</tr>
</tbody>
</table>

**Method of pyrolysis-field ionisation mass spectrometry (Py-FIMS)**

Duplicates of 5 µL of MeOH-dissolved samples were injected into annealed quartz crucibles and dried overnight in a desiccator. Pyrolysis was carried out directly in the ion source of a double-focusing Finnigan MAT95 (emitter: 4.7 kV, counter electrode - 5.5 kV). The samples were heated in a vacuum of 10⁻⁴ Pa from 50 to 700°C in steps of 10°C over a timespan of 15 min. 60 spectra of the mass range 15-900 m/z were recorded at 10 millimasses accuracy. Results were interpreted with the help of marker signals (m/z) of relevant substance groups [Hempfling et al., 1988; Schnitzer & Schulten, 1992; Bochove et al., 1996; Schulten & Leinweber, 1996; Leinweber et al., 2009, 2013].
Chapter 4: A semi-automated, KNIME-based workflow for biofilm assays

Please note: .tar files need to be unpacked (e.g. with 7-Zip) before import into the respective programs.

- **plate-layout-template.xlsx** (available here)
  Plate layout template for recording sample placement in multi-well plates and merging metadata with measurements in KNIME. See Fig. 4.2, p. 53 for illustration.

- **Viaflo-scripts.tar** (available here)
  Viaflo electronic pipetting scripts to successively remove cells, crystal violet staining solution and wash water after steps 1, 3 and 5 (Table 4.1). See Fig. 4.2, p. 53 for experimental context and Vialink’s built-in help for importing instructions.

- **Magellan-readout.mth** (available here)
  Plate-reading method for Tecan’s Magellan software. See section Robotised biofilm quantification, p. 50 for details.

- **KNIME-workflow.tar** (available here)
  Importable workflow for the KNIME Analytics Platform to demonstrate the merging of sample metadata (plate-layout-template.xlsx) and Magellan-measured absorbance data (.asc files). See Fig. 4.1 for illustration. Please note that importing will return an error initially, because the file paths can not match, and have to be corrected as described in section KNIME workflow for data processing. In case of the File Reader nodes, this correction should be conducted with the option Preserve user settings for new location activated. If forgotten, and if the data preview shows a column filled with question marks, please right-click on that column and activate the option DON’T include column in output table. Traffic light symbols below the nodes will indicate whether corrections are still necessary (red), or whether the nodes can be executed (yellow).

  Upon execution of this workflow, data files are read in and the Expand Well Position nodes ensure equal formatting of the sample metadata and measurement results according to the well coordinates (defined in the .xlsx file and present in the .asc files). Joiner combines these tables per row, discarding incongruencies between plate layouts and measurement data. Concatenate progressively merges two plates’ data tables into one. Plate Heatmap Viewer provides a visual comparison of the data processing result with the visual impression of a plate. In particular, the expected locations of biofilm-negative and -positive controls are easily discernible. In the concatenated table, Plate Row Converter and Column Combiner regenerate the alphanumeric well coordinates so that the data and visual impression of individual wells can be compared. Column Filter and Column Resorter exclude obsolete coordinate metadata and pre-format the remaining table for export by CSV Writer.

- **plot-KNIME-output.R** (available here)
  R code to demonstrate the plotting of KNIME-processed data. Please note that due to a randomisation function in the plate layout .xlsx file, editing the latter and running the KNIME workflow and this script again may produce a plot with different assignments of data points to the levels X, Y and Z.

- **plot-Tecan-figures.tar** (available here)
  R code and data (.csv format) used to produce the plots in this article.
Figure 6.12: Detailed instructions for the manual preparation of the *Achnanthidium minutissimum* bioassays, based on the protocol by Windler et al. [2015]. See figure 4.2, p. 53 for method overview.
Chapter 5: Capsules of the diatom *Achnanthidium minutissimum* arise from fibrillar precursors and foster attachment of bacteria.

Figure 6.13: **Identification of *A. minutissimum* cell clusters in axenic culture by subsequent observation by both bright-field (A) and scanning electron (B) microscopy (scale bars: 5 µm).** Demonstration of the same technique used to identify the appearance of xenic biofilms and dehydrated capsule material in SEM (Fig. 5.2, p. 66) in axenic cultures after 31 days of incubation with much fewer adherent cells and no capsules.

Figure 6.14 (following page): **Scanning electron micrographs of fibril-covered *A. minutissimum* frustules from axenic culture.** Samples were prepared for SEM after 20 days of incubation. A (scale bar: 1 µm) & B (scale bar: 200 m): Frustules with few, short fibrils, which were not found in xenic biofilms. C (scale bar: 1 µm) & D (scale bar: 200 nm): Frustule with medium-dense fibrillar mesh, as also seen in xenic biofilm (main Fig. 5.4A). E (scale bar: 200 nm): Fibrils are not only flatly attached to the frustule but also stick out into space and make contact with other cells (arrows), as also seen in xenic cultures (main Fig. 5.4B).
General acknowledgements

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Publications and contributions

The laboratory work for this thesis and the writing was conducted by me with the partial exception of chapter 2 (see below). The abstract was proofread by Dr. Bernard Lepetit. The chapters General introduction and Concluding remarks and perspectives were proofread by Lili Chu and Dr. Ansgar Gruber, respectively, as well as by Prof. Peter G. Kroth. Kai-Uwe Eckhardt provided the supplemental Py-FIMS information. Individual contributions to the chapters are listed below.

Chapter 2: Biofilm and capsule formation of the diatom Achnanthidium minutissimum are affected by a bacterium (published in April 2015 in the Journal of Phycology; 51:343–355; 10.1111/jpy.12280)

- Dr. Miriam Windler designed and performed the experiments, analysed their results and wrote the manuscript.
- Katrin Leinweber contributed equally by reproducing several of those experiments for publication under MW’s supervision, by performing and analysing several supplemental control experiments, as well as by writing their supplemental information.
- Dr. Carolina Rio Bartulos advised about the assembly of S32’s 16S rDNA sequence.
- Prof. Bodo Philipp advised about the design of the experiments and the interpretation of the results.
- All authors proofread the manuscript.

Chapter 3: A bioassay-guided fractionation of bacterial infochemicals that induce biofilm formation by Achnanthidium minutissimum

- Katrin Leinweber designed and performed the experiments, analysed the data, as well as wrote and revised the manuscript.
- Prof. Dieter Spiteller and Prof. Peter G. Kroth discussed the design and results of the experiments, contributed reagents and devices, as well as revised the manuscript.


- Katrin Leinweber conceived and performed the experiments, analysed the data, wrote the manuscript, as well as prepared figures, tables and files for publication.
- Silke Müller designed pipetting scripts, the plate-reading method and KNIME workflow, and contributed the devices, as well as ideas to the experimental design.
- Prof. Peter G. Kroth contributed ideas and revisions to the manuscript.

Chapter 5: Capsules of the diatom Achnanthidium minutissimum arise from fibrillar precursors and foster attachment of bacteria (published in March 2015 in PeerJ 3:e858; 10.7717/peerj.858)

- Katrin Leinweber conceived, designed and performed the experiments, analysed the data, prepared figures, as well as wrote and revised the manuscript.
- Prof. Peter G. Kroth contributed reagents/materials/analysis tools, discussed the design of the project, and reviewed drafts of the manuscript.
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Please note: Some digital object identifiers were shortened for either aesthetic or functional reasons. These shortened DOIs begin with 10/ rather than 10., but resolve in the same way when appended to doi.org/to form a URL.


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