

Diatoms from littoral zone of Lake Constance:  
Diversity, phylogeny, extracellular polysaccharides  
and bacterial associations

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**Rahul A. Bahulikar**  
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1. Referent: Prof. Dr. Peter Kroth  
2. Referent: Prof. Dr. Bernhard Schink



*Dedicated to my beloved father*

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## Chapter 1

### General introduction

### *Biofilms*

‘Biofilms are a collection of adhered cells and their products at a surface or a coating’ or ‘covering on the surface of a living or nonliving substrate composed of organisms like bacteria, protozoa, algae and invertebrate animals’ (O’Toole et al., 2000; Stoodley et al., 2002).

Biofilms are a characteristic feature of water bodies such as streams, wet surfaces and shoreline of rivers, lakes and oceans. They represent miniature ecosystems comprising enormous interactions between different abiotic factors e.g. high energy waves (Hoagland & Peterson, 1990), temperature (Stevenson et al., 1996), water level fluctuations (WLF) (Wei & Chow-Fraser, 2006), light intensity (Hoagland & Peterson, 1990), nutrients (Douglas, 1958) etc. and biotic components like algae, bacteria, fungi and protozoa. Like all other ecosystems, in biofilms, these biotic factors include species from various groups and phyla; which may also interact with each other; it may be competition, symbiotic, parasitic or pathogenic associations (Cole, 1982). These interactions play an important role in the formation, proliferation and / or destruction of the biofilm. The availability of nutrients or changes in the climatic condition becomes unfavorable for one organism and in contrast, favorable situation for another. Some organisms utilize a particular compound and excrete unwanted in the nature, whereas, others use that excreted product as their energy source (Brock & Clyne, 1984). All interactions, drastic environmental changes and also high disturbances caused by human’s leads to complex situations. Who so ever can adapt to most of such fluctuations may have better chances of survival than others.

From all the abiotic factors, seasonal water level fluctuations (WLF) and high-energy waves are the most important factors influencing the epilithic biofilms. Increased water levels can give scope to microorganisms to migrate or to colonize on newly flooded surfaces, whereas decreasing water levels expose the colonised organisms to unfavourable conditions such as desiccation (Robson & Matthews, 2004) and high temperature differences (Steinman & McIntire, 1990), which may eventually lead to the death of the biofilm. Mainly the littoral zone and especially the lake shore is most affected by WLF (Wei & Chow-Fraser, 2006). The substratum near margins of the lake or below shallow shoals is generally composed of rocks of variable sizes or coarse sand. This region is regularly disturbed by high-energy waves

(Stevenson *et al.*, 1996). Generally the upper eulittoral zone is more variable in microhabitats than deeper areas of the lake (Stevenson *et al.*, 1996).

All these interactions among biotic and abiotic factors are very complex. To study them as a ‘whole’ is a mammoth’s task. For our understanding, we break them in to various small pieces and try to take meaning out of it. A comprehensive study of each part and then to connect each other like a jigsaw puzzle then possibly it can give an idea about ‘what’s going on’.

### *Biofilms in the littoral zone*

The first step towards the formation of the biofilm on any surface is the adsorption of an organic layer. This formation of a thin organic film is very fast and inevitable followed by colonization which is the next step towards formation of the biofilm (Cooksey & Wigglesworth-Cooksey, 1995). First, the cells colonizing a new surface or a substrata have easy access to light and nutrients and as the community develops, initial colonizers are covered by additional cells and materials (Cohn & Weitzell, 1996).

Diatoms and bacteria are considered to be the pioneers in developing photic biofilms (Rao *et al.*, 1997) and act as the major primary producers and consumers of organic matter respectively. Previously, bacteria were considered as initial colonizers, followed by diatoms. In a recent report (Ács *et al.*, 2000), coccoid bacterial colonization was observed on the glass slides within three hours of incubation in the water followed by diatoms after six hours. Within 24 h of incubation, the community consisted of a total of 35 species. It was also observed that diatoms show up on stainless steel or on glass surfaces within a few hours of incubation (Cooksey & Wigglesworth-Cooksey, 1995). Under laboratory conditions, diatoms can also attach to the substratum in the absence of bacteria (Cooksey, 1981). Diatoms appear to colonize preferentially on hydrophobic surfaces having intermediate surface energies. Meanwhile, it is accepted that diatoms are the first eukaryotic organisms to colonize on any new surface submerged in the water. However, the presence of bacteria on surfaces can accelerate algal attachment (Gawne *et al.*, 1998) or may have either inhibitory or stimulatory effect on algal growth depending on the taxa involved (Cole, 1982; Hoagland *et al.*, 1993). This biofilms formation is affected by surface topology, surface charges, the presence of organic films, temperature, availability of nutrients and associated species (Gawne *et al.*, 1998).

Dominance of diatoms in periphyton communities has been demonstrated in intertidal mudflats (Cooksey & Wigglesworth-Cooksey, 1995), lakes (Buczko & Ács, 1997; Hawes & Smith, 1994; King et al., 2000), rivers (Ács et al., 2000), streams (Sherwood & Sheath, 1999) etc. Pennate diatoms are often the dominant eukaryotic members of phototrophic biofilms and are early colonizers of natural and artificial substrata (Wetherbee *et al.*, 1998). Many authors showed successional patterns during the formation of biofilm (Ács et al., 2000; Chan et al., 2003; Nandakumar et al., 2004; Sekar et al., 2004; Soininen & Eloranta, 2004). According to Sekar et al. (2004), diatom succession has been influenced by water velocity, size, immigration and reproductive rate of the organism. Biofilm thickness is influenced by the species composition and the season (Sekar *et al.*, 2004). Patric (1973) reported that *Cocconeis* and *Achnanthes* were the first colonizers followed by diatoms like *Fragilaria* and *Synedra*, whereas (Ács & Kiss, 1993) noted that araphid diatoms are pioneer colonist and that small species are fast reproducing and are better competitors in nutrient rich environment (Sekar et al., 2004; Steinman & McIntire, 1990). As the biofilm develops, composition of community may change with the season, the age etc. and finally the 3D structure of biofilm formation was demonstrated (Jackson et al., 2001).

The algal layer is important as a source of organic C, which afterwards is transferred to upper trophic levels. This layer may control the rate and direction of inorganic nutrient exchange between the benthic and pelagic compartments (Underwood & Kromkamp, 1999). The productivity of the attached algae in the freshwater comprised principally of diatoms in the littoral zone may equal or exceed that of pelagic algae (Hoagland et al., 1993; Wetzel, 1964).

### *Extracellular polymeric substances and biofilms*

Extracellular polymeric substances (EPS) are the nonliving mucilaginous material contributing substantially to the fresh weight of biofilms (Sutherland, 2001). Exact nature and texture of biofilms vary with environmental conditions and the composition of microbial community (Hoagland et al., 1993; Sutherland, 2001). The benthic biofilms are formed by secretion of EPS by diatoms and/or bacteria. It forms a matrix or tangle of threads in which the particles from sediment or organisms entangle and form a complex structure (Hoagland et al., 1993; Sutherland, 2001).

The stability of the sediment is depends on the presence of a biofilm (de Brouwer et al., 2005) and this few millimetres of layer supports microbial consortia

and microphytobenthos. These sediments almost always contain high amounts of dissolved organic carbon in their pore water (Cooksey & Wigglesworth-Cooksey, 1995). A positive correlation was shown between sediment stability and various parameters including algal biomass, two types of colloidal carbohydrates and sediment water content. EPS concentration was highly correlated with algal biomass but not with the number of bacteria, suggesting that algae may have a better potential for EPS production than bacteria (Yallop *et al.*, 2000). An increase in the concentration of soluble EPS and bound EPS was observed during the light period and it was decreased during the dark period (Orvain *et al.*, 2003) suggesting that the EPS production was might be of photosynthetic origin. The decrease in EPS concentration in the dark might be due to utilization by diatoms or bacteria to accomplish the energy demands (Stal & Défarge, 2005).

### *Diatoms*

Diatoms are the most diverse and important group of eukaryotic microorganisms on earth and are probably well in excess of 100,000 species (Mann, 1999) and are of immense ecological importance (Falkowski *et al.*, 2004; Smetacek, 1999). They are found in both freshwater and marine environments as well as in moist soil, on wet surfaces, in unusual places like whale skins, in hot springs or highly basic or acidic environments; ice brine canals etc (Mann, 1999). and also occur as endosymbionts in some dinoflagellates (Chesnick *et al.*, 1997) and foraminifera (Chai & Lee, 2000).

Diatoms are important in global nutrient cycling. About 40% of total carbon (Mann, 1999) and silica sub sequestered are fixed by less than few hundred species of the marine plankton (Nelson *et al.*, 1995). Marine food webs and the oceanic biogeochemical cycles are fuelled by the primary production of diatoms (Nelson *et al.*, 1995; Smetacek, 1999).

Diatom fossils are used for stratigraphic calibration, fossil deposits are used as abrasives and filters also as a source of food or valuable biochemical compounds (Cohn & Weitzell, 1996; Falciatore & Bowler, 2002). Fossil diatom frustules (diatomaceous earth) form an indispensable product in washing powder, car polish, tooth paste, dynamite and are also used in filtration of beer, wine sugar, oil and water (Kooistra *et al.*, 2003).

Diatoms are also important components in freshwater lakes, where they comprise a large portion of the total algal biomass (Stevenson et al., 1996). As individual species of diatoms are sensitive to changes in nutrient concentration and pH, the trophic level can be estimated after studying diatom composition (Pouličková et al., 2004). Therefore, they are used as indicators of water quality.

*Taxonomy and phylogeny of diatoms*

The taxonomical and evolutionary relation among diatoms taxa has been studied using two approaches morphology i.e. classical taxonomy by using frustule structures (Fig.1) and molecular phylogenetics (Kooistra et al., 2003). Diatoms are very beautiful organisms when observed under microscope because of their characteristic cell walls (called frustules), made of amorphous silica and consisting of two ornamented compound structures comprising two large valves called as epitheca and hypotheca (Fig. 1) and series of girdle bands on the cingulum. The frustules contain a mesh of small pores for nutrient and gas exchange (Fig. 1). The mesh on the valve is known as striae, in pennates only there may also be a simple or complex slit on the valve called a raphe. The shape of the frustule, structure and number of the raphe, number and structure of pores in the striae, and the presence of a cingulum are used for taxonomic identification of the diatoms.

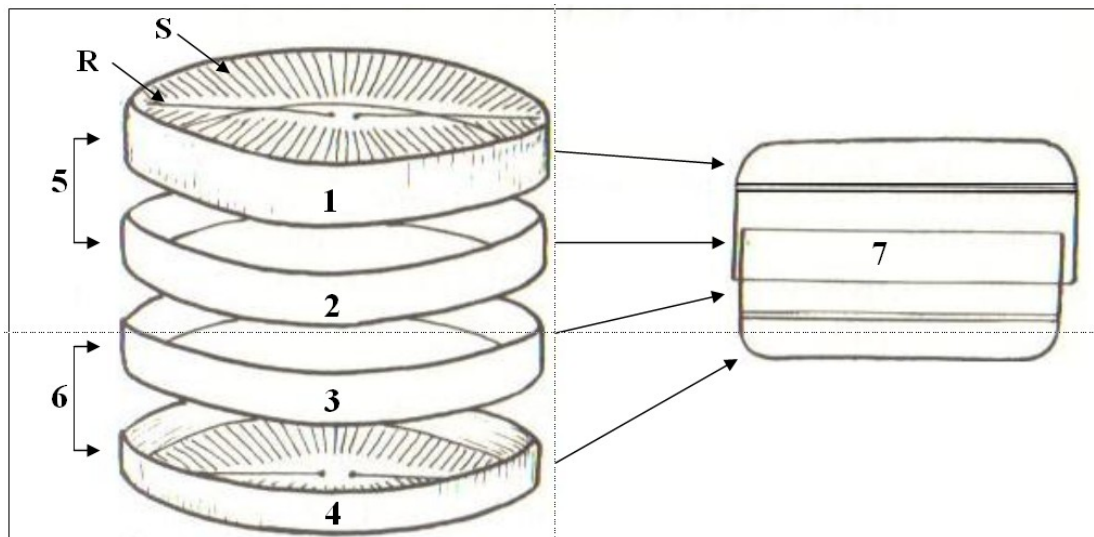


Fig. 1 Diagrammatic presentation of the diatom frustule 1: Face of the epivalve, 2: epicingulum, 3: hypocingulum, 4: hypovalve, 5: epivalve and epicingulum together called as epitheca, 6: Hypovalve and hypocingulum together called as hypotheca, 7: Complete frustule with overlap of the cingula, R: Raphe and S: Striae

Morphologically there are two main groups: centric and pennate diatoms. Centrics are radially symmetrical, whereas pennate diatoms are bilaterally symmetrical and show absence of raphes (araphid) or one or more raphes (raphids) (Kooistra et al., 2003).

Differences within homologous DNA sequences are helpful for the phylogenetic analysis of the organisms (Kooistra et al., 2003). Molecular phylogenies of the diatoms have been studied in great details by using 18S rDNA region (Kooistra & Medlin, 1996; Medlin et al., 1988; Medlin & Kaczmarska, 2004; Medlin et al., 1996; Medlin et al., 1993; Medlin et al., 1996). Several other DNA markers have also been used such as large subunit ribosomal DNA, ITS, *cox A* (Ehara et al., 2000), *Tuf A*, rubisco large subunit (*rbcl*) (Kooistra et al., 2003).

18S rDNA sequence comparison supported a monophyletic origin of diatoms, (Medlin et al., 1993). The centrics showed a paraphyletic origin, whereas pennate diatoms were demonstrated to have monophyletic origin (Medlin et al., 1996). Fossil records of the most ancient deposits and molecular analysis of recent diatoms demonstrated that appearance of centric diatoms was earliest in upper Jurassic at 180 Ma, pennate diatoms at 90 Ma and raphids at 45 Ma. Therefore the phylogeny and fossil record suggests that centrics were the first to evolve followed by araphid pennates and finally raphids pennates (Kooistra *et al.*, 2003). Ultrastructure and life histories were reported to be in accordance with molecular phylogenies (Kooistra *et al.*, 2003).

Based on their habitat, diatoms are either free-floating (planktonic forms) or attached (benthic forms) and colony morphology differs from species to species. On the basis of their habitat, the buoyancy of the cells may be different. Planktonic species are less buoyant and colony morphology is such that the cells can easily float or remain in water column for long time whereas pennets can easily sink (Stevenson et al., 1996). Colony morphology is different in various species; most of them remain single celled, and some species form chain-like colonies with varying chain lengths, and can attach to surface firmly etc.

### *Diatom EPS: secretion and attachment*

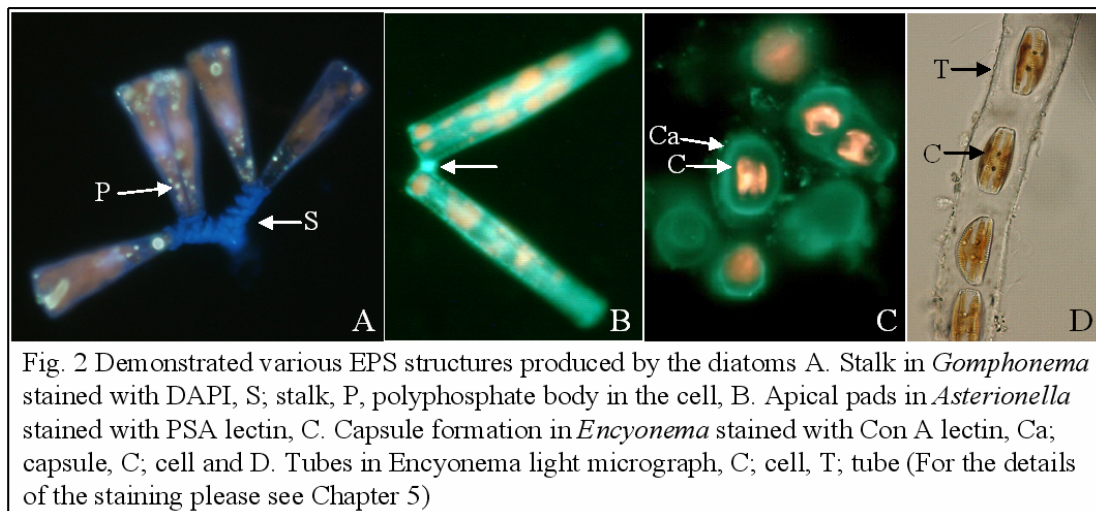
‘Man made adhesives normally fail in wet conditions; however, diatoms produce adhesives that are extremely strong and robust both in freshwater as well as marine environment’ (Gebeshuber *et al.*, 2003).

Freshwater and marine diatoms store the photosynthetically produced carbohydrates as  $\beta$ -1,3-linked glucans (e.g. leucosin, chrysolaminarin) that are used in the dark period (Underwood & Paterson, 2003). Excess of fixed C generally is secreted as EPS. It was reported that ~80% of the fixed C in a diatoms was in excess and was probably transferred mainly in production of EPS (Stal, 2003). This resulted in the speculation that EPS production is a convenient mechanism to divert the excess of light energy, which otherwise can cause oxidative damage (Stal, 2003). The photosynthetic origin of EPS was proved by using  $^{14}\text{C}$  as a tracer in the cultures and demonstrated the presence of radioactivity in the EPS within 3 hours (Smith & Underwood, 2000). In axenic cultures, high EPS accumulation was observed during day light, which decreased during the dark period. It suggests light dependent nature of EPS (Staats et al., 2000). When EPS secretion occurred during the dark period, it was in expense of the intracellular stored carbohydrates and can accelerate within 1-2 h of incubation in light (Smith & Underwood, 2000). This secreted EPS is either just released into the water (or in the medium) and/or used for attachment (locomotion, permanent attachment in the form of stalks, tubes, capsules or pads etc.). Most pennate diatoms secrete EPS through the longitudinal slit present on the silica wall known as raphe or apical pore field in the cell wall while some centric diatoms use axillary pores (Hoagland *et al.*, 1993).

Diatoms possess the ability to attach to the substratum either temporarily or permanently. A temporary attachment includes characteristic diatom movement (gliding), whereas a permanent attachment occurs when diatoms produce various extracellular structures (Hoagland et al., 1993). Gliding and extracellular structure formation is mainly associated with the secretion of extracellular polymeric substances (EPS). Pennate diatoms first attach to the substratum and then they glide; both these processes are accompanied by secretion of EPS (Drum & Hopkins, 1966; Wetherbee et al., 1998) Diatoms can move and vertically migrate during dark and day light (Underwood & Paterson, 2003). EPS secreted in the darkness is proportional to the glucan utilized and its molecular weight was slightly lower than when secreted under illuminated conditions (Smith & Underwood, 2000). This carbohydrate rich adhesive remains as a trail on the surface of the substratum which eventually accumulates as a biofilm (Higgins *et al.*, 2000). Cytoskeleton disturbing drugs were used to investigate the role of actin, myosin and microtubules in diatom gliding and observed that latrunculins (actin inhibitor) and butanedione monoxime (myosin

inhibitor) were the potent and reversible inhibitors of diatom gliding (Poulsen *et al.*, 1999). This provided evidence that diatom gliding is based on actin/myosin motility system (Poulsen *et al.*, 1999).

Permanent attachment is achieved by continuous secretion of EPS. The carbohydrates may form morphological structures classified as cell coatings (silicious frustules with organic layer), adhering sheaths/capsules (EPS material around organic layer), tubes (pseudofilaments around cells), pads (small globular structure attaching cells to other cells or to substratum) or stalks (unidirectionally deposited multilayered structure attaching cells to substratum) (Hoagland *et al.*, 1993).



Stalks are extremely filamentous fibrils and are composed of several layers and with different orientations (Wustman *et al.*, 1997). Stalks are produced from a specific region called apical pore field (APF). In some species, after cell division stalks can form branched structures (Fig 2A). Stalks have a basal attachment disc helping the diatoms to remain firmly attached to the surface (Fig 2A). Because of stalks, diatoms can raise themselves from the surface of the biofilm and still remain attached to the substratum. This strategy might be useful to avoid high competition for nutrients and light. e.g. *Gomphonema*, *Cymbella*, *Achnanthes* etc. (Hoagland *et al.*, 1993).

Apical pads are also called short stalks. Pads are small globular structures mainly useful for cell-to-cell attachment or cell to substratum attachment. Like stalks, the origin of the pads also is the APF region. This attachment type is 5-15 times stronger than stalks. These diatoms can easily remain attached to the substratum exposed to high-energy waves, in streams e.g. *Synedra*, *Diatoma*, *Asterionella* etc (Fig 2B) (Hoagland *et al.*, 1993).

Adhering films/capsules are the amorphous secretion present around the cells (Fig 2C). Unlike stalks, origin of the films is ascertain and capsule secretion is observed in many taxa (Hoagland et al., 1993).

Tubes are the long EPS structures around the cells. Diatoms can remain motile within tubes (Fig 2D). Depending on age, the rigidity of the tube varies. Younger tubes are delicate, whereas older tubes are rigid and branched. Secretion of tube like structures might occur by girdle pores. Tube formation has been described in several diatom genera like *Encyonema* (*Cymbella*), *Berkeleya* (Hoagland et al., 1993).

Cell coatings are the organic layers around the cell wall and are firmly attached to the cell wall. It might be useful to avoid silica dissolution. Cells coatings are observed in many diatom taxa (Hoagland et al., 1993).

EPS secretion is useful for diatoms in various ways: 1. physical connection during conjugation, 2. limit digestion by fish or other organisms, 3. act as nutrient collection apparatus, 4. to reduce osmotic stress, 5. to unite pregametangial cells during sexual reproduction, 6. to minimize desiccation etc. (Hoagland et al., 1993).

### *Labelling of EPS and microscopy*

*In-situ* localization of EPS components becomes important for visualization and characterization. Previously, cytochemical methods and electron microscopy were used by (Daniel *et al.*, 1987) to study EPS localization of 17 marine diatom species. Use of several stains were found to be useful to investigate the chemical nature of the EPS like polysaccharide (periodic acid-Schiff test), sulphated polysaccharide (alcian blue staining), presence of proteins (dinitrofluorobenzene), lipids (Sudan black B) etc. (Daniel et al., 1987).

Lectins are proteins or glycoproteins of non-immune origin. They bind to the carbohydrates specifically but reversibly and agglutinate cells or precipitate glycoconjugates (Song *et al.*, 1999). When coupled with fluorochromes, the specific affinity of the lectins becomes a useful tool to detect the presence of specific sugar moieties in the EPS. FITC conjugated-lectins were used to differentiate various toxic species of *Pseudo-Nitzschia* from New Zealand based on their differential production of surface sugars and also reported that these changes in surface sugars depend on geographical origin and/or environmental conditions (Rhodes, 1998). In a recent report, lectins with fluorescent markers were used to differentiate several extracellular

polymers produced by marine biofilm diatoms like *Navicula* and *Amphora* and were helpful in studying cell-cell interactions (Wigglesworth-Cooksey & Cooksey, 2005).

Other fluorophores like DAPI (4',6-diamidino-2-phenylindole) and DTAF (5-(4,6-dichlorotriazinyl) amino fluorescein) were also used for localization. DAPI is well known for its DNA binding properties. It also binds to polysaccharides by an unknown mechanism. Negatively charged polyelectrolytes and dextran sulfate (a sulfated glycan) form a fluorescing complex with DAPI, yielding a blue emission. DAPI is also useful to detect the presence of polyphosphate deposition in the cells (Kawaharasaki *et al.*, 1999; Wustman *et al.*, 1997). DTAF reacts directly with polysaccharides and peptides at room temperature at a pH above 9, thus it is useful for labelling natural organic compounds without disturbing its natural form (Schumann & Rentsch, 1998).

#### *Chemical extraction and analysis of EPS*

Carbohydrates are the main components of EPS (Staats *et al.*, 1999; Stal & de Brouwer, 2003), and may also contain very small amounts of proteins (Staats *et al.*, 1999), glycoproteins (Chiovitti *et al.*, 2003; Lind *et al.*, 1997) and uronic acids (Chiovitti *et al.*, 2003; Chiovitti *et al.*, 2003; de Brouwer & Stal, 2002; Staats *et al.*, 1999). Many diatoms are able to secrete very large amounts of EPS in nature as well as in cultures especially in the stationary phase (de Brouwer & Stal, 2002; de Brouwer *et al.*, 2002; Staats *et al.*, 1999).

Substratum adhesion and gliding also involved the presence of proteoglycans and antisera raised against frustule-associated proteins also detected proteins within the raphe, the cell surface and the gliding trail. (Lind *et al.*, 1997). These glycoproteins are associated mainly with short-chained oligosaccharides containing xylose, galactose, rhamnose, mannose (Chiovitti *et al.*, 2003).

Isolation of unique polysaccharides requires optimization of the extraction and fractionation protocol according to diatom species and nature of EPS (Chiovitti *et al.*, 2003). Chiovitti *et al.* (2003) used hot water, hot NaHCO<sub>3</sub> and NaOH with NaBH<sub>3</sub> for sequential extraction of bound polysaccharide in the freshwater diatom *Pinnularia viridis*. The extraction procedure was monitored using Atomic Force Microscopy (Chiovitti *et al.*, 2003).

### *Diatom-bacterial Interactions*

Diatom and bacteria are the dominant members of the biofilm community in the littoral zone of Lake Constance. In addition, both are considered as primary colonizers. As diatoms are the major primary producers, the study of associated bacteria could also reflect their probable role in natural biofilms in degradation of complex organic matter produced by the diatoms. Interaction between the different organisms is the essential for formation, maintenance and / or degradation of the biofilm. EPS secreted by diatoms might be an important energy source for heterotrophic bacteria (Girollo *et al.*, 2003). More than 50% of  $^{14}\text{C}$  labelled EPS of natural benthic community and EPS from 2 species of diatoms were utilized by bacterial community in the sediment within 24 h of incubation (Goto *et al.*, 2001).

Axenic diatom cultures are difficult to establish and to maintain for long time, whereas non-axenic cultures can be maintained for long time. These cultures harbor a distinct assemblage of associated bacteria, which also have been termed as satellite bacteria (Schäfer *et al.*, 2002). As such cultures are maintained for several years, the associated bacteria may undergo selection. Satellite bacteria associated with marine and freshwater diatoms have been studied previously and were found to be mainly members of CFB phylum and  $\alpha$ -proteobacteria (Makk *et al.*, 2003; Schäfer *et al.*, 2002). Bacterial succession was reported in diatom-dominated aggregates and in marine snow of planktonic origin. Knoll *et al.* (2001) demonstrated that primarily  $\alpha$ -proteobacterial communities were present, and then  $\beta$ -proteobacteria and finally the *Cytophyga*, *Flavobacteria* and *Bacteriodis* (CFB) group became dominant. In other studies, reintroduction of planktonic bacterial community in the axenic diatom cultures and subsequent observation of bacterial community dynamics revealed a selection process to favor the growth of a specific group and elimination of remaining bacteria during diatom growth (Grossart *et al.*, 2005). This diversity also suggests that different phytoplankton species may require various specific bacteria (Schäfer *et al.*, 2002) or diatom growth might depend upon specific bacterial compounds like vitamin B<sub>12</sub> (Croft *et al.*, 2005). As the bacteria are known to degrade macromolecular dissolved organic matter and utilize C of small molecular mass, it was hypothesized that bacteria might be responsible for degradation of EPS secreted by diatoms.

Diatom attachment is a very complex phenomenon. As bacteria are also primary colonizers, specific interaction might be present to facilitate or inhibit diatom attachment. Gawne *et al.* (1998) showed the attachment of *Achnanthes longipes* on

hydrophobic surfaces like polystyrene is inhibited by bacterial biofilms whereas on other surfaces, bacterial biofilms either facilitate or have no effect on diatom attachment. Under axenic condition diatoms showed evenly distributed growth whereas after addition of bacteria diatoms showed clump formation and eventual death of diatoms was observed (Wigglesworth-Cooksey & Cooksey, 2005). From these experiments, it is clear that there is diatom-bacterial association. However, exact nature of the association is not yet clear.

### *Study site: Lake Constance*

Lake Constance is a mesotrophic, phosphorous limited and hard water prealpine lake (Schmieder *et al.*, 2005) and lies to the north of the Alps at 395 m above sea level. Lake Constance has a surface area of 476 km<sup>2</sup>, its maximum and mean depth is 252 m and 110 m, respectively (Rosenstock & Simon, 1993), and has a volume of 48.5 km<sup>3</sup>. The lake basin is situated in the Molasse basin of the northern Alpine foreland and was mainly formed by water and ice activity during the last quaternary glaciation period more than 15.000 years before present. The catchment area of Lake Constance is about 11.5 km<sup>2</sup> and covers the territories of three European countries: Germany (28%), Switzerland with Liechtenstein (48%) and Austria (24%). The lake is divided into the lower lake and the upper lake (Schmieder *et al.*, 2005). More than 90% of the water flow originates from the Alps by the three inflows Alpenrhein, Bregenzer Aach and Dornbirner Aach in the eastern part of the Upper Lake. In Lake Constance, the water levels may fluctuate in average 2 m in altitude per year. As a natural ecosystem, Lake Constance is a representative and significant natural habitat for plants and animals in Central Europe.

Algal and bacterial diversity in the pelagic zone of Lake Constance has been studied in great details over the past decades (Knoll *et al.*, 2001; Schweitzer *et al.*, 2001; Sommer, 1984; Sommer, 1985; Zwisler *et al.*, 2003). Since the first decade of oligotrophication, the concentration of phosphate was decreased substantially, resulting in a major change with respect to the composition of the organisms. Sommer *et al.* (1993) demonstrated the dominance of the planktonic diatom species *Asterionella formosa* Hass., *Fragillaria crotonensis* Kitton, and *Stephanodiscus binderanus* Krieger and also studied the population dynamics of these three species (Sommer, 1984) including seasonal succession (Sommer, 1985). Wessels *et al.* (1999)

described the history of eutrophication in the lake and showed changes in diatom population structure from pelagic zone.

There are many reports available on the bacterial communities growing in lake snow particles, which are known to be dominated mainly by diatom aggregates. The bacterial community structure was analyzed in water column samples that were taken at various depths. This analysis showed the abundance of  $\alpha$ - and  $\beta$ -proteobacteria at 25 m depth, whereas  $\beta$ -proteobacteria dominated in samples from 50 & 110 m depth (Schweitzer *et al.*, 2001). A bacterial succession was demonstrated in macroscopic organic aggregates at laboratory conditions sampled from Lake Constance (Knoll *et al.*, 2001). They showed first a dominance of  $\alpha$ -proteobacteria, while after 24 h incubation time the number of  $\beta$ -proteobacteria increased and finally the CFB group became dominant after 48 h.

### *The main objectives of this thesis*

1. To study the effect of various water levels on the diatom and bacterial community structure from epilithic biofilms of the littoral zone of Lake Constance (Chapter 2).
2. To study seasonal fluctuations in the diatom community structure in epilithic biofilms and EPS dynamics (Chapter 3)
3. Isolation, identification and phylogenetic analysis of various diatoms from epilithic biofilms from Lake Constance (Chapter 4)
4. To characterize diatom secreted EPS by localization (Chapter 5) and fractionation approach (Chapters 6 and 7)
5. To study diatom-bacterial interactions (Chapter 8)

## Chapter 2

# Diatom and bacterial community structure of epilithic biofilms from littoral zone of Lake Constance

Rahul A. Bahulikar and P. G. Kroth\*

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Fachbereich Biologie, University of Konstanz, 78457 Konstanz, Germany.

Key words: epilithic biofilm, bacterial abundance, diatom diversity, EPS,

Abbreviations: quantitative PCR: qPCR, EPS: extracellular polymeric substances,  
HGC: High GC content gram positive, LGC: Low GC content gram positive, CFB:  
*Cytophaga/Flavobacteria/Bacteroidetes*

### **Abstract**

We have analyzed the community structure of diatoms and bacteria in epilithic biofilms from the littoral zone of Lake Constance by classical methods (for diatoms) and by characterizing the bacterial community structure using taxon specific quantitative PCR (qPCR) assays. Samples were collected at five locations being 50 m apart from each other and at each location at depths of 20, 30, 40 and 50 cm. In total, 110 different diatom species from 21 genera were identified by light microscopy. Shannon's and Simpson's diversity indices showed moderate diatom diversity, and PCA analysis of diatom communities demonstrated a depth-wise grouping. qPCR was performed to study the abundance of six taxa of bacteria:  $\alpha$ -,  $\beta$ - proteobacteria, the *Cytophaga – Flexibacter - Bacteroidetes* group (CFB), high GC content (HGC) gram positives- *Actinobacteria*, low GC content (LGC) gram positive bacteria and the *Acidobacteria* group. Our studies revealed a dominance of  $\beta$ -proteobacteria, CFB and HGC bacteria in these biofilms. We also analyzed the amounts of soluble carbohydrates and chlorophyll *a* within all sampled biofilms, showing that both parameters correlated significantly. Generally, in samples collected at a depth of 50 cm chlorophyll *a* and carbohydrates values were lower, while the number of diatom species and the percentage of  $\beta$ -proteobacteria were higher compared to samples collected at lower depths. High negative correlation between  $\beta$ -proteobacteria and soluble EPS amounts also suggests their possible degradative role in mature biofilms.

### **Introduction**

Lake Constance is a mesotrophic, phosphorous limited and hard water prealpine lake (Schmieder et al., 2005). The lake undergoes a yearly water level change cycle, in which the shoreline in the littoral zone is dry during winter and gets flooded in spring. Our studies focus on such a shoreline from the littoral zone where epilithic biofilms or periphyton communities are found. These biofilms are a characteristic feature of aquatic habitats e.g. streams, rivers, lakes and oceans. They represent miniature ecosystems comprising enormous interactions between different biotic components i.e. algae, bacteria, fungi, protozoa etc. and abiotic factors e.g. waves, temperature, changing water levels, nutrients etc. From all the abiotic factors, increased water levels give scope to microorganisms to migrate or to colonize on new surfaces whereas decreasing water levels expose the settled organisms to unfavourable conditions such as desiccation, which can eventually lead to death of the biofilm. Mainly the littoral zone and especially the lake shore is the most affected region due to water level fluctuations (WLF) (Wei & Chow-Fraser, 2006). This shallow upper littoral zone of lentic periphyton communities is often dominated by diatoms and green algae (Stevenson et al., 1996) This part experiences regular high energy wave disturbances and is normally dominated by taxa that are capable of attaching firmly to the substratum (Stevenson et al., 1996).

Diatoms and bacteria are the pioneers in developing photic biofilms (Rao et al., 1997) and act as the major primary producers and consumers of organic matter, respectively. Productivity of the attached algae in freshwater comprised primarily of diatoms in the littoral zone may equal or exceed that of free-floating algae (Wetzel, 1964). Therefore, diatoms are important component of food web in streams, lakes and marine environment across a wide range of pH, nutrient and physical conditions (Hoagland et al., 1993). They are the major producers of extracellular polymeric substances (EPS) in the biofilms which mainly consist of carbohydrates (Hoagland et al., 1993; Underwood & Paterson, 2003). The EPS matrix is important for maintaining the infrastructure of the biofilm, and also helps to capture nutrients (Rao et al., 1997). Apart from being an attractive source of nutrients for heterotrophic bacteria, it increases sediment stability, a substratum for other organisms and acts as an anti-desiccant to prevent drying of the biofilm where surfaces are exposed periodically to air (Hoagland et al., 1993)

Algal and bacterial diversity in the pelagic zone of Lake Constance has been studied in great detail over the past decades (Knoll et al., 2001; Schweitzer et al., 2001; Sommer, 1984; Sommer, 1985; Zwisler et al., 2003), demonstrating a dominance of the planktonic diatom species *Asterionella formosa* Hass., *Fragillaria crotonensis* Kitton, and *Stephanodiscus binderanus* Krieger. Sommer et al. (1993) showed effects of oligotrophication on the changes in the phytoplankton diversity and the population dynamics of these three species including seasonal succession (Sommer, 1985). Wessels et al. (1999) described the history of eutrophication in the lake. In Lake Constance, planktonic bacterial communities are active in other metabolic hotspots such as lake snow or microaggregates, have been studied by molecular methods such as FISH, DGGE (Knoll et al., 2001; Zwisler et al., 2003).

However, the communities of benthic diatoms and bacteria growing on epilithic biofilms from Lake Constance have not been well studied. The epilithic or stone biofilms are dried up as water levels decline in the winter and are recolonised by bacteria, diatoms, algae, fungi, protozoa etc. during reflooding in spring. Assuming that the biofilms at lower depths would be relatively younger than the biofilms at higher water depths, the increasing water levels could provide a natural time series for studying community structure of the biofilms. Thus, our main aim was to study the benthic diatom and bacterial community structures in epilithic biofilms and to analyze any patterns of changes in community structure across the small depth gradient in reflooded areas.

To study the abundance of six different taxa of bacteria, we used qPCR assays specific for the detection of  $\alpha$ -proteobacteria,  $\beta$ -proteobacteria, CFB (*Cytophaga-Flavobacterium-Bacteroidetes*), high GC content gram positive (HGC) - *Actinobacteria*, and low GC content gram-positive (LGC) bacteria and *Acidobacteria*, which covered the majority of the eubacteria.

### **Materials and methods**

#### *Study area and sampling strategy*

Sampling was carried out in the littoral zone of Lake Constance (Germany, 47°41'N, 9°11'E). To measure the effect of the small-scale depth gradient and the community structure of the diatoms and bacteria, one large or three to four small stones adjacent to each other were collected from five different locations and named A to E. These five locations are lying in a row and the distance between two adjacent locations was 50 m). Biofilms growing on the upper surface of stones were collected on April 15, 2005. To study the effect of water depth on the diatom diversity, the biofilms were collected at depths of 20, 30, 40 and 50 cm at each location and referred as sites. Adhering biofilms were scraped off from the stones and stored in Falcon tubes. Pore water was removed by centrifugation at 6000 rpm for 10 minutes. The resulting pellets were divided into three parts. One part was frozen in liquid nitrogen and subsequently used for DNA extraction. The second part was used for chlorophyll assays where chlorophyll *a* was determined according to the method of Jeffrey & Humphrey (1975), and the last part was used for identification and counting of diatoms. The supernatant/pore water was used for measuring concentration of soluble carbohydrates using glucose as standard (Dubois et al., 1956).

#### *Diatom preparation and counting*

Fresh samples were checked for diatom viability (>80%) before H<sub>2</sub>O<sub>2</sub> treatment. Samples for diatom analysis were incubated at 95°C for 3 hrs in 10% H<sub>2</sub>O<sub>2</sub>, followed by overnight incubation in 10% HCl at room temperature, where carbonates were removed (Battarbee, 1986; Battarbee et al., 1999). To remove residual HCl the samples were washed twice with distilled water. Cleaned samples were suspended in distilled water and mounted in Naphrax (Euromex Microscopes, Netherlands). From each sample, 400 diatom frustules were identified and categorized according to Krammer & Lange-Bertalot (1986-1991). Broken frustules and diatoms that were mounted on their girdle side were not considered for identification and counting. Diatoms were observed at 1000X magnification using an Olympus microscope (BX51) equipped with a Nikon digital camera (DMX-1200). Diatom cells were identified to the highest taxonomic resolution before counting.

### *DNA extraction and qPCR*

Around 40-50 mg of biofilm was used for extracting DNA. A modified CTAB method (Murray & Thompson, 1980) was used and yielded DNA of good quality. Dried pellets of DNA were dissolved in 50  $\mu$ l of TE buffer (10 mM Tris/Cl, pH 8.0, 1 mM EDTA). For exact quantification of DNA, we used the SYBR Green I assay (Zipper et al., 2003) and DNA was diluted to a concentration of 1ng  $\mu$ l<sup>-1</sup>.

qPCR was used to detect the abundance of specific phylogenetic groups of microorganisms using taxon specific primers for 'all groups'(eubacteria),  $\alpha$ -proteobacteria,  $\beta$ -proteobacteria, CFB, HGC - *Actinobacteria*, LGC and *Acidobacteria* (Fierer et al., 2005). qPCR was performed in 96 well plates in an ABI 7500 real-time PCR system with the ABI Prism ® 7000 sequence detection system v1.2.3 (Applied Biosystems, Darmstadt). Each reaction of 20  $\mu$ l included Power SYBR® green PCR master mix (10 $\mu$ l) with ROX as a passive reference (Applied Biosystems, Darmstadt), 10  $\mu$ M of each primer and 1.0 ng of template DNA. PCR steps included 10 min initial denaturation (95°C) followed by 40 cycles of denaturation (15 s at 95°C), annealing and extension at 60°C (except for the eubacterial assay where annealing was 53°C) for 30 s. Detection of primer-dimers was avoided by keeping the data acquisition temperature at 80°C for 35 s. This temperature was selected based on the melting curves. Each sample and standards were analysed in triplicates. Standards were prepared using group specific bacterial strains as follows:  $\alpha$ -proteobacteria – *Hyphomicrobium aestuarii* (DSM-1564),  $\beta$ -proteobacteria – *Azorcus anaerobius* (DSM-12081), CFB group - *Flavobacterium aquatile* (DSM 1132), HGC - *Actinobacteria Arthrobacterium capsulatum* (DSM 11244), LGC - *Bacillus licheniformis* (DSM 13) and *Acidobacteria* group - *Acidobacter crystallopoietes* (DSM 20117). 16S rDNA regions were amplified from these bacterial strains using 27f and 1492r primers. After quantification, cleaned PCR products were serially diluted and used as standards.

### *Data analysis*

Simpson's, Shannon's diversity indices, evenness and principal component analysis (PCA) were calculated using the MVSP software (Kovach, 2002). qPCR results were analyzed using MS excel. The number of target molecules per ng of DNA (standard) were calculated assuming that the average molecular mass of the

double-stranded DNA molecule is 660 g/mol (Fierer et al., 2005). A range of standards ( $10^8$  target molecules to  $10^2$  target molecules per reaction) was used. After the qPCR run, standards showed a linear relationship between the log of the plasmid DNA copy number and ct values at specific concentration ( $R^2 > 0.97$  in each case). Numbers of target molecules or copies per ng of biofilm DNA were calculated from the standard graphs, related to the eubacterial copy numbers, and expressed as relative abundances.

## Results

Samples were taken in April 2005. Data on water level fluctuations were obtained from the Institute of Limnology, University of Konstanz (Germany). According to these records and personal observations the water level in Lake Constance increased drastically by 39 cm between March and April 2005, whereas the increase of the water level was rather low from February to March and January to February, i.e. 10 cm and 7 cm, respectively. Accordingly, the 50 cm water depth area in our study was flooded in February - March, the 40 cm area in March, whereas the sites of 20 cm and 30 cm were flooded between March and April. Diatom diversity and abundance were measured in biofilms collected from Lake Constance at different sites and at different depths labelled as A-E according to their position and affixed number <sub>20-50</sub> according to the depth. In this analysis, we were able to identify a total of 110 different diatom species belonging to 21 genera. The majority of them were pennate diatoms (>99%), whereas centric diatoms were represented by only one genus, *Cyclotella*, with total frustule count of 15 (which is 0.19 % of the total counts). At each site, total diatom species counts varied from 32-52 with an average of  $40.95 \pm 5.34$  species/site (Annexure 1). *Fragilaria* was the most dominant genus comprising 19 different species, followed by *Cymbella* (17 species), and *Achnanthes* (16 species). If we consider the total frustule number, *Fragilaria fasciculata* (For authorities and species list please refer Annexure 1) and *F. capucina* were the dominant species with an average of 62.65 and 41.75 frustules per count per site. Diatom species with a count of more than 100 frustules in total were considered as dominant species.

In almost all locations, the number of species counted at depths of 50 cm was relatively higher than at other depths at the same location, however, no progressive increase in species number was observed. Progressive decrease in frustule number with increase in depth was demonstrated for *Achnanthes minutissima* (location A), *Diatoma vulgare* (A-C) and *Denticula tenuis* (A & B), and a progressive increase was observed for *Amphora inariensis* (A and E), *Cymbella minuta* (A), *Fragilaria brevistriata* (A), *F. capucina* (E) and *F. pinnata var. pinnata* (A) (Annexure 1)

Shannon's and Simpson's diversity indices ranged from 2.52 (D<sub>30</sub>) - 3.08 (A<sub>30</sub>) and 0.87 (D<sub>30</sub>) - 0.94 (A<sub>30</sub> and C<sub>50</sub>), respectively indicating a moderate diatoms diversity. Evenness values ranged from 0.73 (D<sub>30</sub>) - 0.84 (A<sub>30</sub>) (Fig. 1A, B) suggesting a rather homogenous distribution.

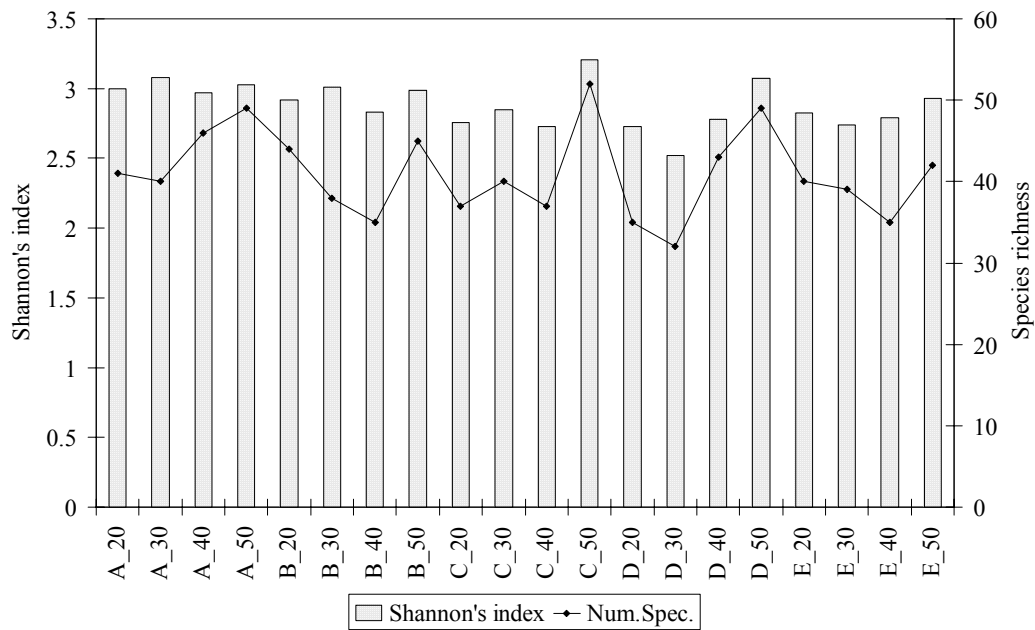


Fig 1A: Values of Shannon's index (columns) and species richness (dots) of diatoms from epilithic biofilms from littoral zone of Lake Constance taken at 5 different sites (A-E) and at 4 different depths (20-50 cm).

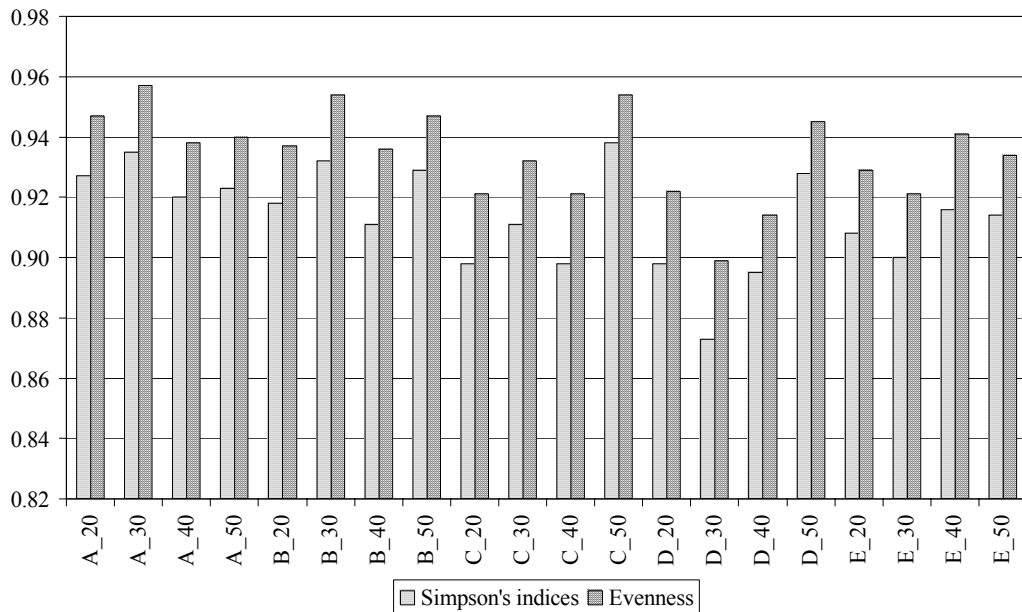


Fig 1B. Simpson's diversity indices (column with dots) and evenness (column with bricks) from the same samples as in A.

Spatial patterns were revealed by Principal Component Analysis (PCA), which showed the presence of three main groups and two outliers (A<sub>50</sub> and E<sub>40</sub>). Group I consists of 3 samples from locations A, E, from 20-40 cm depth and all samples of D. Samples from B and C forming Group II and 50 cm samples from B and C clustered together forming Group III (Fig 2). Group I and Group III showed a close relation

between samples from the same depth (e.g. A<sub>20</sub> and E<sub>20</sub>; D<sub>30</sub> and E<sub>30</sub> and B<sub>50</sub> in Group I, B<sub>30</sub> and C<sub>30</sub>; and C<sub>50</sub> in group III).

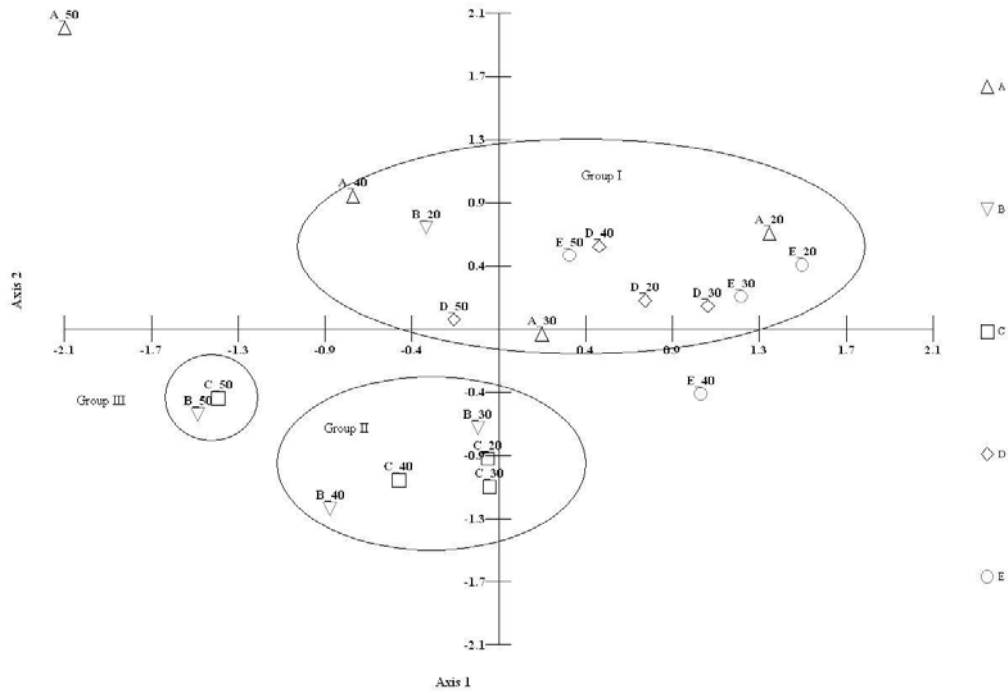


Fig 2: PCA ordination plot for diatom samples with the diversity of diatoms across collection sites from littoral zone of Lake Constance for same samples as in Fig 1A. Here A-E are the locations and suffixed number represents depth in cm.

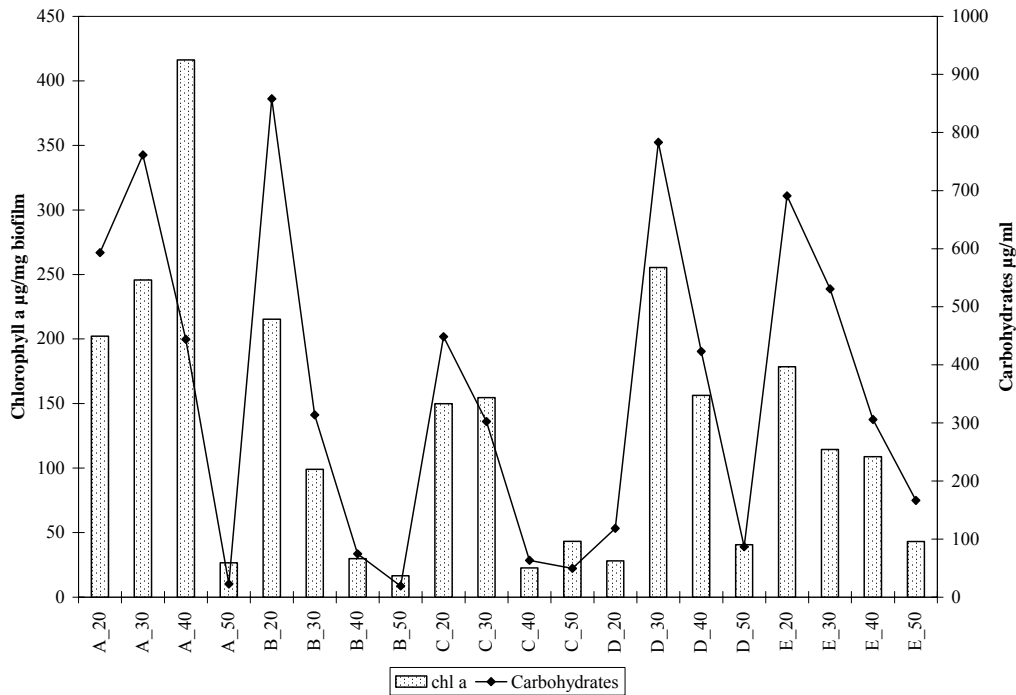


Fig 3: Chlorophyll *a* (Columns) and soluble EPS content (dots) measured in epilithic biofilms from Lake Constance taken at indicated sites (A-E) at different depths.

Chlorophyll *a* contents ranged from 16.4 (B<sub>50</sub>) to 416.3  $\mu\text{g l}^{-1}$  (A<sub>40</sub>). Location A showed the highest amount of chlorophyll *a* whereas in samples from location B the chlorophyll *a* content was rather low (Fig. 3). Generally, at 20 cm depth we found higher chlorophyll *a* contents (except for D<sub>20</sub>). The amount of soluble carbohydrates varied within sites (Fig. 3). Very high carbohydrate concentrations were found at one site (B<sub>20</sub> 858.2  $\mu\text{g ml}^{-1}$ ) while the lowest value was observed at site sample B<sub>50</sub> (16.6  $\mu\text{g ml}^{-1}$ ) (Fig 3). In all locations, the samples collected at 50 cm depth showed the lowest concentrations of soluble carbohydrates. The chlorophyll *a* and carbohydrate values were highly correlated ( $R^2 = 0.77$ ) with each other, whereas, no correlation was observed between species richness per location and either the chlorophyll *a* or EPS concentrations.

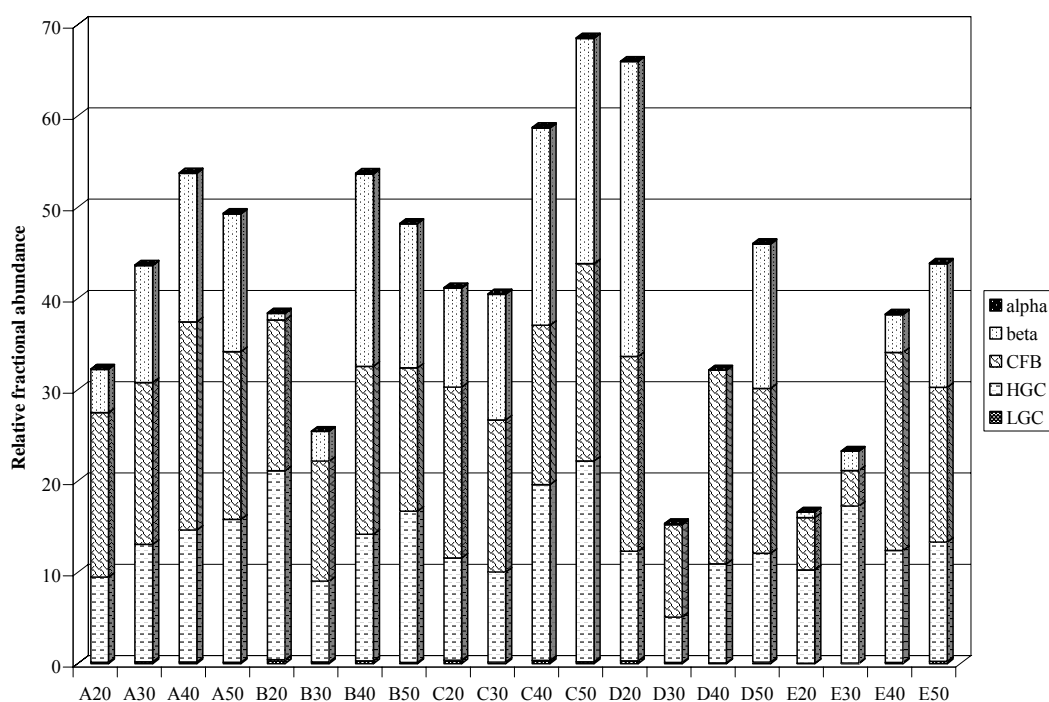


Fig 4: Relative abundance of  $\alpha$ -,  $\beta$ -proteobacteria, *Cytophaga / Flavobacteria Bacteroidetes* group (CFB), High GC content gram positive – *Actinobacteria* (HGC) and Low GC content gram positive group (LGC) compared with eubacterial copy number (16S rDNA) from epilithic biofilms of Lake Constance estimated by qPCR. Samples were taken at indicated sites (A-E) at different depths.

Relative abundance of the bacteria was estimated for six phylogenetic groups by qPCR that comprised 16.61% - 68.56% of the total bacteria (Fig. 3). Significantly higher abundances of the  $\beta$ -proteobacteria, CFB and HGC groups compared to the  $\alpha$ -proteobacteria, LGC and *Acidobacteria* group was found. Estimated relative

abundance of  $\alpha$ -proteobacteria and LGC were lower than 1 % of the total eubacterial numbers (Fig 4). The average abundance of  $\beta$ -proteobacteria, CFB and HGC groups were 11.46 %, 16.71% and 13.36%, respectively. *Acidobacteria* were generally below the detection level.  $\alpha$ -proteobacteria and CFB bacteria showed a moderate negative correlation ( $r^2 = -0.42$  and  $-0.51$  respectively), while the  $\beta$ -proteobacteria revealed a high negative correlation with the EPS content ( $r^2 = -0.71$ ).

### Discussion

The littoral zone of Lake Constance revealed a high diversity and richness of diatom species in the epilithic biofilms. Interestingly, species richness was higher at 20 cm depth and showed a slight decrease at middle depths, followed by higher species richness again in deeper areas. This trend was observed in almost all locations (Fig. 1). When compared with water level data, biofilms at lower depths were still developing and facing high disturbances due to waves compared to deeper sites. The 50 cm biofilms were thicker and appeared relatively thick and mature as compared to the biofilms at lower depths (Bahulikar, personal observation).

Among the most abundant genera in all the depths were *Fragilaria*, *Achnanthes*, and *Cymbella* species as well as *Diatoma vulgare* and *Fragilaria* sp. are also susceptible to displacement and colonies can be easily broken and suspended by the water currents and observed to have faster recovery than other diatoms (Peterson et al., 1990). Generally, colonies of *Fragilaria* form long chains that do not attach firmly to the surface. This peculiar dispersal mechanism might be responsible for its presence in such high abundance within our samples. *Achnanthes* was represented by 16 different species, but only *A. minutissima* was actually dominant. This is a small diatom observed to have very high growth rate (Peterson et al., 1990), which can recover from disturbances caused by waves. This diatom was also found to be a highly dominant periphyton species in Lake Velencei (Ács & Buczkó, 1994). Complex interactions may occur in different components of biofilm communities such as intra- and inter-species competition for resources or grazing. All of these factors can contribute to the overall community structure (Stevenson et al., 1996). Nevertheless, we observed some interesting patterns of increase in diatom species abundance at some sites whereas, as discussed above, *Fragilaria* species are capable of easy displacement and thus can be easily dispersed by waves and are found in higher numbers at low water depths.

Other dominant genera we found were *Gomphonema* and *Cymbella*, which mostly consists of stalk producing species. Stalks are developed from the unidirectional secretion of EPS helping the diatoms to attach firmly to the substratum (Hoagland et al., 1993) so that they might not be easily dispersed by water currents. *Fragilaria* and *Cymbella* sp. are capable of rapid colonization and in our study, they were found at high numbers. Grazing is also an important factor, which results in

removal of communities. *Achnanthes*, *Cymbella* and *Synedra* have been reported to be disturbance and grazing resistant species (Stevenson et al., 1996).

Diversity indices like Shannon's and Simpson's express the richness and variation in natural ecological communities, while evenness expresses the abundance of the species in the sample or in community (Tsirtsis & Karydis, 1998). Kingston et al. (1983) reported a higher range of Shannon indices (2.57-3.74) for benthic diatom diversity from Lake Michigan, a large oligotrophic lake. The value range for the Shannon index in this study was slightly lower than that of Lake Michigan, suggesting the presence of moderate diatom diversity in the benthic biofilms of Lake Constance.

Principal component analysis revealed three groups, which demonstrates a close relation between different locations and the same depth. All collection sites were in one row and the distances between two adjacent sites were about 50 m, which is very large for migration of epilithic algae. Diatom components were similar in sites further apart with respect to depth. Depth-wise clustering was observed, which indicates a relatedness of samples from the same depth and different locations.

Biomass of microphytobenthic communities can be measured by analysing the chlorophyll *a* content of the biofilms. In our study, chlorophyll *a* concentrations were relatively higher in the samples of lower depth than samples from deeper sites. In Lake Velencei and Danube River, significant differences were found in the species composition, abundance and chlorophyll *a* content of epiphyton at different depths, but the transparency of these waters was lower than Lake Constance (Barreto et al., 1997; Buczkó & Ács, 1997). The same pattern was shown by soluble EPS. A significant correlation between algal biomass and EPS concentration has been reported previously, suggesting that the soluble EPS might be produced by the benthic diatom community (Staats et al., 2001; Sutherland, 2001; Underwood & Smith, 1998). In accordance with this, our data also showed a strong correlation ( $R^2 = 0.77$ ) between chlorophyll *a* and soluble EPS content. At the same time, there was no EPS detectable in open water (data not shown), suggesting that EPS was mainly restricted to biofilms. This observation is in accordance with Sutherland (2001), who reported that EPS is uniquely and specifically synthesized in biofilms. For EPS production, the composition of the phototrophs is also important. It has been shown that diatom dominated biofilms with mixed taxa produce significantly high amounts of soluble EPS (Underwood & Paterson, 2003) compared to cyanobacteria-dominated biofilms (Bellinger et al., 2005). As in our data there was no correlation between individual

diatom species counts and EPS concentration, many diatom species might be responsible for its production.

The bacterial community structure of epilithic biofilms in Lake Constance has not been studied until now, although there are many reports on the bacterial communities in other metabolic hotspots like lake snow or diatom microaggregates. Of the six different eubacterial taxa examined,  $\alpha$ -proteobacteria,  $\beta$ -proteobacteria, CFB and HGC constitute for the majority of bacteria in many other freshwater systems (Gao et al., 2005). In our study, the high abundance of CFB and HGC - *Actinobacteria* was observed in almost all the depths and sites. Interestingly, we observed that the  $\beta$ -proteobacterial abundance increased with depth and showed a high negative correlation with soluble EPS, which reflects that this group might be particularly responsible for degradation of the soluble EPS or became dominant when the soluble EPS components were degraded and established themselves in mature biofilms (at 40 or 50 cm). The site D<sub>20</sub> was an exception, which showed very different features, as compared to other 20 cm sites, i.e. low chlorophyll, low EPS and very high percentage of  $\beta$ -proteobacteria. In this case, the  $\beta$ -proteobacteria might be responsible for the degradation of soluble EPS keeping its value low.

The CFB group was one of the co-dominating taxa and also showed a moderate negative correlation with EPS content ( $r^2 = -0.41$ ). Members of the CFB group are known to have the ability to hydrolyze complex polysaccharides of different compositions, e.g., cellulose or chitin, which are rather difficult to degrade for other bacteria (Kirchman, 2002). CFB members can also utilize DNA, lipids and proteins released mainly from dead organism in the biofilm (Kirchman, 2002). The bacteria can also swarm or glide on surfaces and are known to form swarming colonies. Biofilms formed on stones thus are ideal surfaces for the proliferation of these bacteria. Some CFB members also are filamentous which helps them to escape from grazing (O'Sullivan et al., 2002).

According to this study, HGC - *Actinobacteria* are a further dominant group in the epilithic biofilms. However, there was no correlation observed with either EPS or chlorophyll *a*. It is known that freshwater *Actinobacteria* are globally distributed in the limnic systems (Allgaier & Grossart, 2006) and constitute a major fraction of heterotrophic bacterioplankton. Their small size and their rigid cell wall structure may enable them to escape grazing (Allgaier & Grossart, 2006). Although their ecological

role is poorly understood (Allgaier & Grossart, 2006) their spreading hyphae-like morphology (as CFB members) might make them successful colonizers on stones.

Bacterioplankton studies from Lake Constance at 3 m depth, following a phytoplankton bloom showed a dominance of  $\beta$ - proteobacteria (34 $\pm$ 10%), the CFB group constituted (19 $\pm$  8%) and  $\alpha$ - proteobacteria (14 $\pm$ 8%) (Zwisler et al., 2003). Compared to this, our study of the epilithic biofilms from the littoral zone revealed an overall dominance of CFB, HGC- *Actinobacteria* and  $\beta$ - proteobacteria, while  $\alpha$ - proteobacteria were present of a negligible percentage. Studies done on microaggregates from Lake Constance also had revealed a dominance of  $\beta$ - proteobacteria and the CFB group and similar to our study,  $\alpha$ - proteobacteria were not detected at all. Specifically the diatom microaggregates were dominated exclusively by  $\beta$ - proteobacteria (upto 60%) (Brachvogel et al., 2001) which is very high compared to that in our samples. Hence, it can be summarized that the planktonic and benthic bacterial communities in Lake Constance differed, in their composition.

Although we did not study the relative abundance of other groups such as  $\gamma$ - proteobacteria, *Verrucomicrobia* or *Planctomyces* because real time PCR assays for these groups have not been standardised yet, the six groups we studied, together contributed for a major eubacterial population, i.e., a maximum up to 69 % and an average of 40% of the total eubacteria. Interestingly, the total contribution of the CFB,  $\beta$ - proteobacteria and HGC - *Actinobacteria* groups together contributed to a less extent to the biofilms at lower depths, as compared to the biofilms at higher depths (Fig. 4).

Progressive monitoring of the bacterial biofilm development on artificial substrata has been reported by several authors (Ács, 1998; Downes et al., 2000; Jackson et al., 2001; Patrick, 1976; Sekar et al., 2004). According to biofilm formation model proposed by Jackson et al. (2001), three major successional changes take place during biofilm development, an initial stage characterised by colonization of different populations and lack of a structured community, a second stage when few populations dominate, and a mature biofilm stage with complex spatial structure that facilitates greater diversity (Jackson et al., 2001). There are few reports on biofilm models where the succession of primary producers and bacteria has been studied together. The water level data in our study indicated that the areas at which we collected the 50 cm samples was reflooded around a month before the areas of 20 or 30 cm, which themselves were reflooded just a few days before sampling. If we

assume that the biofilm develops as the water level increases, the time span between each depth studied would be in terms of days or months, sufficient for comparison. Based on our data, the following interpretations appear justified: The chlorophyll *a* content and soluble EPS content decreased according to depth, indicating that in young biofilms the primary production was the important process, which led to a higher soluble EPS production. However, the abundance of  $\beta$ -proteobacteria, CFB and HGC – *Actinobacteria* together increased with depths, indicating that these bacterial communities were getting more and more established in mature biofilms at deeper areas, which are known to be specialised in degradation of organic matter and are known to dominate diatom microaggregates (Brachvogel et al., 2001).

In conclusion, we observed significant differences in the community structure of diatoms and bacteria in the epilithic biofilms. We studied parameters such as soluble EPS content in these biofilms, which actually forms a link between the primary production and heterotrophic bacteria. We used the increasing water levels as a natural time scale and studied the trends in two important components of epilithic biofilms, i.e. diatom and bacteria, across a depth gradient. The increasing water level provided us a natural time scale and allowed to study the trends in two important members of epilithic biofilms, i.e. diatom and bacteria, across a small depth gradient.

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**Annexure 1:** Distribution of various diatom species across the five studied sites and each depth.

Species name \Site name	A				B				C				D				E			
	20 cm	30 cm	40 cm	50 cm	20 cm	30 cm	40 cm	50 cm	20 cm	30 cm	40 cm	50 cm	20 cm	30 cm	40 cm	50 cm	20 cm	30 cm	40 cm	50 cm
<i>Achnanthes cf. chlidanos</i>	2												2						1	1
<i>Achnanthes clevei</i> Grunow		1	1	3	1	1	1			1		2			1	1		1		3
<i>Achnanthes delicatula</i> (Kützing) Grunow														1	1					
<i>Achnanthes cf. disaper</i>			2	1																
<i>Achnanthes exigua</i> Grunow			2	3	1			4	1		1	5			1	5		1	1	2
<i>Achnanthes flexella</i> (Kützing) Brun	1	2	4	2	3							1		3		2	1	1		1
<i>Achnanthes helvetica</i> (Hustedt) Lange-Bertalot		1	5	1	1		1	2	1		1	3		2	3		1	2		8
<i>Achnanthes holsaticus</i> Hustedt		1	2	5								1			1					
<i>Achnanthes hungarica</i> (Grunow) Grunow		5	7	14	3	5	6	4	6	5	12	7	2		5	3	1	1		6
<i>Achnanthes ingratiiformis</i> Lange-Bertalot				1																
<i>Achnanthes lanceolata</i> (Brébisson) Grunow			2	4	4	3	2	2		1	11	7	2	1	2	3		3	1	2
<i>Achnanthes lutheri</i> Hustedt															1		1			

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<i>Achnanthes minutissima</i> Kützing	39	34	23	9	14	12	7	12	25	26	16	14	24	25	44	19	46	63	54	46
<i>Achnanthes</i> cf. <i>nitiforma</i>											1	1								
<i>Achnanthes semiaperta</i> Hustedt				1	1							1								
<i>Achnanthes</i> sp.				5	2	1	3	5	2	1		1				3				
<i>Amphora inariensis</i> Krammer	9	18	22	40	17	11	29	29	27	11	13	15	27	5	14	7	3	7	7	24
<i>Amphora ovalis</i> (Kützing) Kützing								1			1				1					
<i>Caloneis silicula</i> (Ehrenberg) Cleve	1																			
<i>Cocconeis</i> cf. <i>neominuta</i>								1								1				
<i>Cocconeis placentula</i> Ehrenburg		3	1	4	2	3				3	1	1	2	1	2	3	2	2		3
<i>Cyclotella bodanica</i> Grunow				1		1				1		1						1		
<i>Cyclotella</i> cf. <i>cyclopuncta</i>						1				1										
<i>Cyclotella</i> sp.1			4	1				1								1				1
<i>Cymbella</i> cf. <i>acyl</i>																				1
<i>Cymbella affinis</i> Kützing			1		1							2			1	1				1
<i>Cymbella caespitosa</i> (Kützing) Brun	1	5	4	3	3			3	2		1	3	2		3	2	1		1	2
<i>Cymbella</i> cf. <i>amphicephala</i>	2			1							1								1	
<i>Cymbella cistula</i> (Ehrenberg) Kirchner		3	1	1		3		2	1	3	2	1				1				1

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<i>Cymbella cymbiformis</i> Agardh	2	1		1							1		1		2				3	
<i>Cymbella</i> cf. <i>falaisendis</i>				1																
<i>Cymbella</i> cf. <i>incerta</i>			1	2																
<i>Cymbella microcephala</i> Grunow	16	20			1	13	7	7		1		7	1	2	3	6	11	23	13	7
<i>Cymbella minuta</i> Hilse	7	17	20	32	21	23	15	54	13	23	41	38	18	6	11	24	7	10	7	16
<i>Cymbella rainhardtii</i> Krammer						1	1	1	1	1								1		
<i>Cymbella silesiaca</i> Bleisch	9	7	3	7	3	7	5	1	7	7		5	9	6	9	21	3	2	6	9
<i>Cymbella</i> cf. <i>sinuate</i>	1																			
<i>Cymbella</i> sp.								1	2		3	2					1		1	
<i>Cymbella subequalis</i> Grunow														1						
<i>Cymella helvetica</i> Krützing		1				1					1									
<i>Cymella lanceolata</i> (Ehrenberg) Kirchner		1			2	1					1		2		1		2	1		2
<i>Denticula</i> sp.					2		1		2					1	4	4	10		1	5
<i>Denticula tenuis</i> Kützing	16	13	1		20	16	3	1	6	9	5	1	34	27	17	58	41	71	23	3
<i>Denticula thermalis</i> Kützing	7			1	3								1	3	1		5	2		5
<i>Diatoma ehrenburgii</i> Kützing	4	3	4	1		3	4	1	3	15	8	5	1	1	2	1		3	14	5
<i>Diatoma vulgare</i> Bory	41	15	7	4	74	40	9	1	22	10	9	9	28	95	98	32	86	21	50	30
<i>Epithemia</i> cf. <i>frickel</i>									1											

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<i>Fragilaria alpestris</i> Krasske																					2
<i>Fragilaria brerolinensis</i> (Lemmermann) Lange-Bertalot																					2
<i>Fragilaria brevistriata</i> Grunow	13	30	35	56	57	36	43	50	19	30	30	21	22	6	22	12	4	8	3	19	
<i>Fragilaria</i> cf. <i>capensis</i>																					1
<i>Fragilaria capucina</i> Desmazières	31	43	54	14	21	58	75	22	64	58	65	49	37	40	38	32	22	25	40	47	
<i>Fragilaria constricta</i> Ehrenberg																					1
<i>Fragilaria construens</i> (Ehrenberg) Grunow	3	6	5	18	11	6	9	20	1	6	4	10	2	2		7		1	7	4	
<i>Fragilaria elliptica</i> Schumann	4	9	13	16	11	9	14	14	8	9	10	9	6	4	3	8	2	3	1	2	
<i>Fragilaria exigua</i> Grunow																					1
<i>Fragilaria faciculata</i> (Agardh) Lange-Bertalot	73	59	66	25	21	45	62	39	92	82	88	51	101	86	49	64	35	69	63	83	
<i>Fragilaria lapponica</i> Grunow					2	1															
<i>Fragilaria</i> cf. <i>leptostuensis</i>																					4
<i>Fragilaria leptostauran</i> (Ehrenberg) Hustedt	3	2	5	10	4	6	6	5	1	2		4	2		3		1	2		2	
<i>Fragilaria parasitica</i> (W.Smith) Grunow																					2
<i>Fragilaria pinnata</i> Ehrenberg	23	36	51	69	41	33	24	40	25	32	26	42	25	3	14	20	10	7	7	15	
<i>Fragilaria pseudoconstruens</i> Van Landingham				2	3									2	1	1	2		2		2

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<i>Fragilaria robusta</i> Hustedt					8			2				7								
<i>Fragilaria</i> sp.								6												
<i>Fragilaria zeilleri</i> Héribaud	1	3	2	9	1	5	7	11	5	3	6	8		1	5	2		1	3	
<i>Gomphonema</i> cf. <i>acutiusculum</i>											3									
<i>Gomphonema angustatum</i> (Kützing) Rabenhorst	5																			
<i>Gomphonema angustum</i> Agardh	4	5			13	8	1	4	1	5	2	1	10	9	1	3	26	14	19	1
<i>Gomphonema augur</i> Ehrenberg	3																			
<i>Gomphonema clavatum</i> Ehrenberg	17	8	1	3					8				10	23	8	1	25	17	17	
<i>Gomphonema mexicana</i> Grunow				2																
<i>Gomphonema olivaceum</i> (Hornemann) Brébisson	18	8	5	3	18	3		6	3	7	1	5	2	17	2	4	9	5		5
<i>Gomphonema parvulum</i> Kützing												2				2				
<i>Gomphonema</i> sp.							6	2	3		2									
<i>Gomphonema truncatum</i> Ehrenberg	2	2	3	1		3	6	3	1	1	2	7			2	1				1
<i>Hantschia marina</i> (Donkin) Grunow											1									
<i>Navicula capitatoradiata</i> Germain	2	3	1		3	6		1	2	3	1	1	3	2	1	1	2		1	2
<i>Navicula</i> cf. <i>angusta</i>						1					1									

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<i>Navicula cf. cupsida</i>	2		1	1															2
<i>Navicula cf. memiculus</i>																			2
<i>Navicula cf. rainhardtii</i>																			1
<i>Navicula cf. schoenfeldii</i>																			1
<i>Navicula cinta</i> (Ehrenberg) Ralfs	4	5	1		1							1	1	1	6	5	5		
<i>Navicula cocconeiformis</i> Gregory					1														
<i>Navicula constans</i> Hustedt											1								
<i>Navicula cryptocephala</i> Kützing	17	3	7	8	1	6		2	2	1	2	10	10	11	5	10	9	5	6
<i>Navicula cryptotenella</i> Lange-Bertalot									1		1								
<i>Navicula placentula</i> (Ehrenberg) Grunow				1	1														
<i>Navicula radiosa</i> Kützing		2			2	5	7	3	7	2	5	6			7	1			
<i>Neidium dubium</i> (Ehrenberg) Cleve								1		1									2
<i>Neidium bisulcatum</i> (Lagerstedt) Cleve																1			
<i>Nitzschia angustata</i> Grunow				2				1			1					1			
<i>Nitzschia cf. aequorea</i>										1									
<i>Nitzschia cf. recta</i>				1										3					
<i>Nitzschia dissipata</i> (Kützing)	1	4						1							1	1			1



## Chapter 3

### Seasonal fluctuations of epilithic diatoms and extracellular polymeric substances from the littoral zone of Lake Constance

Rahul A. Bahulikar and Peter Kroth

Faculty of Biology, University of Konstanz, University str. 10, Konstanz Germany

Key words: chlorophyll *a*, benthic diatoms, EPS,

Abbreviations: bEPS, bound EPS, cEPS, colloidal EPS; eEPS, EDTA extractable EPS; EPS, extracellular polymeric substance;

### **Abstract**

The seasonal succession of diatom communities and the concentrations of extracellular polymeric substance (EPS) were examined in epilithic biofilms from the littoral zone of Lake Constance (Germany). A total of 94 different diatom species from 21 genera were identified and counted from samples taken during June 2004 to June 2005. *Denticula tenuis*, *Achnanthes minutissima* and *Cymbella microcephala* were among the most dominant members. Shannon's index revealed a moderate diversity of diatoms throughout the year. Principal component analysis detected a distinct seasonal pattern in the diatom community structure. Moreover, *A. minutissima*, *D. tenuis*, *C. microcephala* and *Amphora inariensis* showed seasonal patterns. Chlorophyll *a* contents of the biofilms were highly correlated with the pore water EPS and with the bound EPS. The EDTA extractable EPS fractions, which contain EPS tightly bound either to the cells or to sediment particles concentration of which was moderately correlated with the chlorophyll *a*. A high density of photoautotrophic organisms according to chlorophyll *a* measurement (0.2 µg/mg biofilm) observed during April 2005. The diatom community structure and EPS in the epilithic biofilms were strongly influenced by seasonal changes. Increase in water levels and temperature in spring in addition to yearly water mixing of the lake resulted in a high richness of diatom species and a high EPS content.

### **Introduction**

Algal diversity in the pelagic zone of Lake Constance has been studied in great detail over the past decades (Sommer, 1984; Sommer, 1985; Wessels et al., 1999) demonstrating the dominance of various planktonic diatom species. Seasonal succession (Sommer et al., 1993) and population dynamics (Sommer, 1984) of planktonic algae has also been reported earlier. Wessels et al. (1999) described the history of eutrophication by analysing fossilized diatoms from the pelagic sediment of the lake. However, to our knowledge, there are no reports available on the seasonal succession in benthic diatoms growing on epilithic biofilms from Lake Constance.

Epilithic biofilms are characteristic features of habitats such as streams, rivers, lakeshores and oceans. The lakeshore is mainly affected by high-energy waves, changing water levels, fluctuations of the temperature and the light intensity (Wei & Chow-Fraser, 2006). All these factors may have direct or indirect effects on the biofilms. High-energy waves can disturb the biofilms and loosely attached biota may suspend into the open water (Peterson et al., 1990). Considering the impact of water level fluctuations, increased water levels can give scope to microorganisms to migrate or to colonize on newly submerged surfaces, whereas decreasing water levels expose the settled organisms to unfavourable conditions such as desiccation (Steinman & McIntire, 1990).

The productivity of the attached algae in the littoral zone of freshwater habitats may be equal or more than that of pelagic algae (Hoagland et al., 1993; Wetzel, 1964). Epilithic communities contribute significantly to the productivity of the littoral zone of the lakes (Hawes & Smith, 1994). In the eulittoral zone, diatoms are the major components of the epilithic biofilms (Stevenson et al., 1996). They fix CO<sub>2</sub> during photosynthesis and the excess of fixed carbon is secreted out of the cells in the form of extracellular polymeric substances (EPS) as a metabolic overflow (Stal, 2003). Benthic diatoms can divert up to 80% of the fixed carbon into EPS (Goto et al., 2001). EPS is related to the life cycle of diatoms in various ways. Characteristic diatom movement (gliding) is directly associated with EPS secretion. Diatoms may form EPS structures like stalks, pads, tubes and capsules (Hoagland et al., 1993; Underwood & Paterson, 2003). EPS also plays an important role in the formation, maintenance of the infrastructure of the biofilms and help in capturing nutrients (Rao *et al.*, 1997). Stability of the sediment is governed by the presence of EPS (Bellinger

et al., 2005; de Brouwer & Stal, 2001; Staats et al., 2001; Underwood & Paterson, 1993; Yallop et al., 2000).

Stones are considered as ‘inert’ substrata for colonization by benthic algae (Stevenson et al., 1996). Unlike sediments, the epilithon represents a firm and stable substratum. In epilithic biofilms, EPS is mainly useful for maintaining biofilm integrity and capturing nutrients (Stevenson et al., 1996). Here we report on the seasonal fluctuations in diatom communities and changes in EPS concentration from epilithic biofilms of Lake Constance.

In this study, we focus on the following questions:

- 1) Do epilithic diatoms from Lake Constance show seasonal fluctuations?
- 2) Do changing water levels have any effect on the species richness?
- 3) Are there any seasonal changes in the concentrations of EPS in epilithic biofilms and does it show any correlation with the diatom communities?

### **Materials and methods**

#### *Study area*

Lake Constance is a mesotrophic, phosphorous limited, hard water prealpine lake (Schmieder *et al.*, 2005). The lake is divided into two parts the lower lake and the upper lake with a total surface area of 476 km<sup>2</sup> and maximum depth of 252 m (Rosenstock & Simon, 1993). In Lake Constance, the seasonal water levels may fluctuate on an average of 2 m in altitude per year, spring water level starts to increase in March. Maximum water level is normally reached in June or July. After that, water level starts to decline again and the minimum water level is attained in January or February.

#### *Sampling strategy*

To measure the seasonal changes in the diatom community from epilithic biofilms, 3-4 small stones were collected every time at depths of ~20-30 cm from Jun 2004 to Jun 2005 at two places, which were 50 m apart from each other from the littoral zone of Lake Constance, Germany (47°41'N, 9°11'E). In the laboratory, adhering biofilms were scraped off from the stones and pooled for each collection site. Pore water was removed by centrifugation at 5000 rpm for 10 minutes and was stored at -20 °C. The resulting pellets were further divided into three parts. One part was used for the identification and documentation of diatom frustules (described below). One part was used for determination of chlorophyll *a* according to the method of Jeffrey & Humphrey (1975), and one part was used for the analysis of EPS (as described below).

#### *Sample preparation and community analysis*

Fresh samples were checked microscopically for diatom viability (>80%) before H<sub>2</sub>O<sub>2</sub> treatment. Samples for diatom analyses were incubated at 95 °C for 3 hrs in 10% H<sub>2</sub>O<sub>2</sub>, followed by overnight incubation in 10% HCl at room temperature to remove carbonates (Battarbee, 1986; Battarbee *et al.*, 1999). To remove the residual HCl samples were washed twice with distilled water. Cleaned frustules were suspended in distilled water and mounted in Naphrax (Euromex Microscopes, Netherlands). From each slide, 400 diatom frustules were identified and categorized according to Krammer & Lange-Bertalot (1986-1991). Broken frustules and diatoms, which were mounted on their girdle side, were not considered for identification and counting. Diatoms were observed at 1000X magnification using an Olympus

microscope (BX51) equipped with a Nikon digital camera (DMX-1200). Diatom cells were identified the highest taxonomic resolution before counting.

#### *EPS isolation and quantification*

Warm water-soluble EPS was extracted suspending ~30-40 mg of the biofilm pellet (after extraction of pore water) in 1 ml distilled water and was incubated at 30 °C for 1 h with shaking. After centrifugation, the supernatant was stored at -20 °C until further use. The resulting pellet was resuspended in 1 ml of 0.1 M EDTA and was incubated at room temperature (20-22 °C) for 16 h (de Brouwer & Stal, 2001; Stal, 2003). After centrifugation, the supernatant was stored at -20 °C. All three fractions namely pore water (colloidal EPS = cEPS), 30 °C (bound EPS = bEPS) and EDTA extractable EPS (eEPS) were analysed by carbohydrate assays using glucose as a standard (Dubois et al., 1956).

#### *Data analysis*

Simpson's, Shannon's diversity indices, evenness and principal component analysis (PCA) was calculated using the MVSP software (Kovach, 2002). Quantification of EPS was done on the basis of standard curves of glucose using Microsoft excel.

## Results

Seasonal fluctuations and community dynamics of diatoms were studied in epilithic biofilms collected from Lake Constance at two nearby locations. This analysis revealed a total of 94 different diatom species belonging to 21 genera. The majority of them were pennate diatoms (>99%), centric diatoms were represented by *Cyclotella*, *Melosira* and *Stephanodiscus* with a total frustule count of 32 only (which is 0.35 % of the total frustule counts). Among the 94 species, only 16 reached up to >1% of the total frustule count and only five species were more abundant than 5%. At each site, the total diatom species richness varied from 23-43 with an average of  $32.63 \pm 5.66$  species per site.

Table 1 Values of Shannon's, Simpson's diversity indices and Evenness of diatom communities from the littoral zone of Lake Constance. Sample preparation and calculations are described in "Materials and Methods".

Locations	Shannon's Diversity	Simpson's Diversity	Evenness	Richness
Jun 04-S1	2.45	0.87	0.89	28
Jun 04-S2	2.77	0.89	0.92	40
Jul 04-S1	1.79	0.75	0.78	23
Jul 04-S2	2.11	0.80	0.83	28
Aug 04-S1	2.46	0.88	0.91	31
Aug 04-S2	2.31	0.84	0.86	32
Sept 04-S1	2.41	0.85	0.87	35
Sept 04-S2	2.06	0.81	0.84	23
Oct 04-S1	2.39	0.86	0.88	28
Oct 04-S2	2.61	0.89	0.91	35
Nov 04-S1	2.40	0.85	0.87	37
Nov 04-S2	2.59	0.88	0.89	38
Dec 04-S1	2.76	0.91	0.94	34
Dec 04-S2	3.03	0.94	0.96	34
Jan 05-S1	2.69	0.89	0.92	36
Jan 05-S2	2.68	0.89	0.92	36
Mar 05-S1	2.38	0.83	0.86	33
Mar 05-S2	2.97	0.93	0.95	39
Apr 05-S1	2.99	0.93	0.95	41
Apr 05-S2	2.91	0.92	0.94	43
May 05-S1	2.07	0.78	0.81	25
May 05-S2	2.12	0.81	0.84	27
Jun 05-S1	2.24	0.79	0.82	29
Jun 05-S2	2.35	0.85	0.88	28



Fig. 1 Seasonal dynamics of 10 benthic dominant diatom species recorded from June 2004 to June 2005 from two sites of epilithic biofilms from Lake Constance (Site 1 and Site 2). X-axis represents month of biofilm collection and Y-axis shows number frustules per counting. Scales of Y-axis are identical for single species across two sites.

*Achnanthes* was the most dominant genus comprising 17 different species, followed by *Cymbella* (15 species), while *Fragilaria* and *Navicula* contributed with 13 species each. If we consider the total frustule number, *Denticula tenuis* (for authorities please see Chapter 2 Annexure 1), *Achnanthes minutissima* and *Cymbella microcephala* were the most dominant species with an average of 70.0, 64.5 and 51.7 frustules (representing 15-20% of the frustules per counting) respectively per count and time point.

Species richness was variable for each site with respect to time. The minimum number of species were observed in samples from July 04 at Site-1 and Sept 04 at Site-2 (23 species each) and maximum number of species were determined in samples from April 05, (Site-1, 41 and Site-2, 43 species) at both sites. Shannon's index and Simpson's diversity ranged from 1.8 – 2.9 and 0.78 – 0.96 (Table 2). Some species-specific successional trends were observed in the species abundance. Such as in *D. tenuis*, lower frustule count was found in June 04 and a gradual increase was observed until, it reached to the maximum in Aug 04. After that, there was a reduction in number until Apr 05 and finally it again reached to maximum in May 05 (Fig. 1). Similar patterns were observed in *A. minutissima*. However, the third dominant species, *C. microcephala*, was observed to increase in abundance from June 04 and reached its maximum in Sept 04. After some smaller fluctuations in autumn, finally it decreased to a very few frustule counts in winter. Some species like *C. minuta* (Jun 04) and *Diatoma ehrenburgii* (Mar 05) were dominant only for a particular time point (Fig. 1).

PCA analysis revealed a temporal fluctuation pattern. In most cases, collections made in the same month at Site-1 and Site-2 remained very close to each other showing a close relation among them (except from Aug 04, Nov 04) (Fig. 2).

Changing water levels showed a slight negative correlation with species richness and was moderate at Site-1 ( $r^2 = -0.67$ ) and was weak at Site-2 ( $r^2 = -0.35$ ) i.e. low species richness when water levels were high i.e. in summer and vice versa. When the frustule counts of individual species were compared with water level data, *A. minutissima* ( $r^2 = 0.50$  &  $0.51$  Site-1 and Site-2 respectively) and *Cymbella minuta* ( $r^2 = 0.52$  &  $0.45$  respectively) showed a moderate positive correlation. A moderate negative correlation was observed for species like *Amphora inariensis* ( $r^2 = -0.58$  &  $-0.74$  Site-1 and Site-2 respectively) and *D. ehrenburgii* ( $r^2 = -0.45$  &  $-0.68$  Site-1 and

Site-2 respectively). In contrast, *Fragilaria pinnata* from Site-2 demonstrated a very high negative correlation ( $r^2 = -0.73$ ) with water levels.

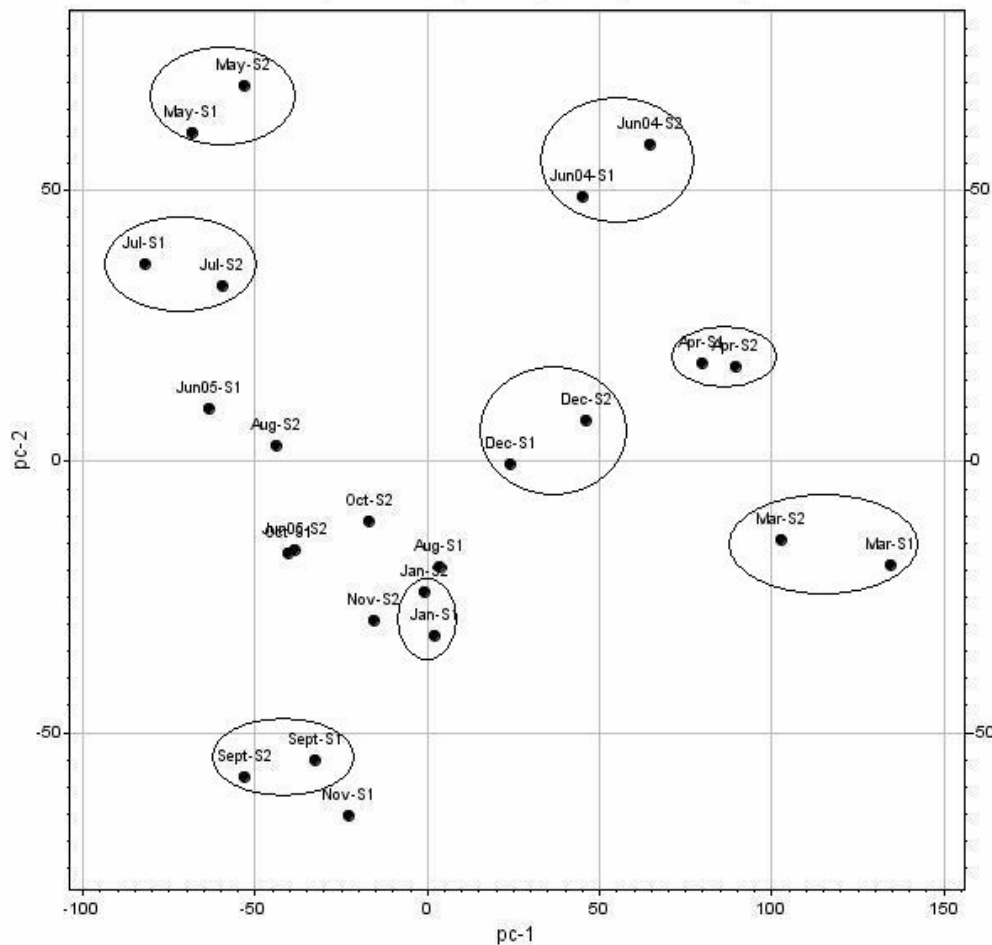


Fig. 2 Principal component analysis scatter plot showing seasonal successional changes in diatom community from two sites (S1 and S2) collected from epilithic biofilms of Lake Constance. Most samples collected in same month are grouped together and are circled

Chlorophyll *a* contents ranged from 0.053 – 0.17  $\mu\text{g}/\text{mg}$  of biofilm. Biofilms from Site-2 showed the highest level of chlorophyll *a* content in Apr 04, whereas, it was lowest in Dec 04 at Site-2 (Fig. 3). An increase in chlorophyll *a* content was observed in the biofilms from both sites in Apr 04 and it decreased again in May 05. Differences in chlorophyll *a* content were observed at both sites during Oct 04 to Mar 05.

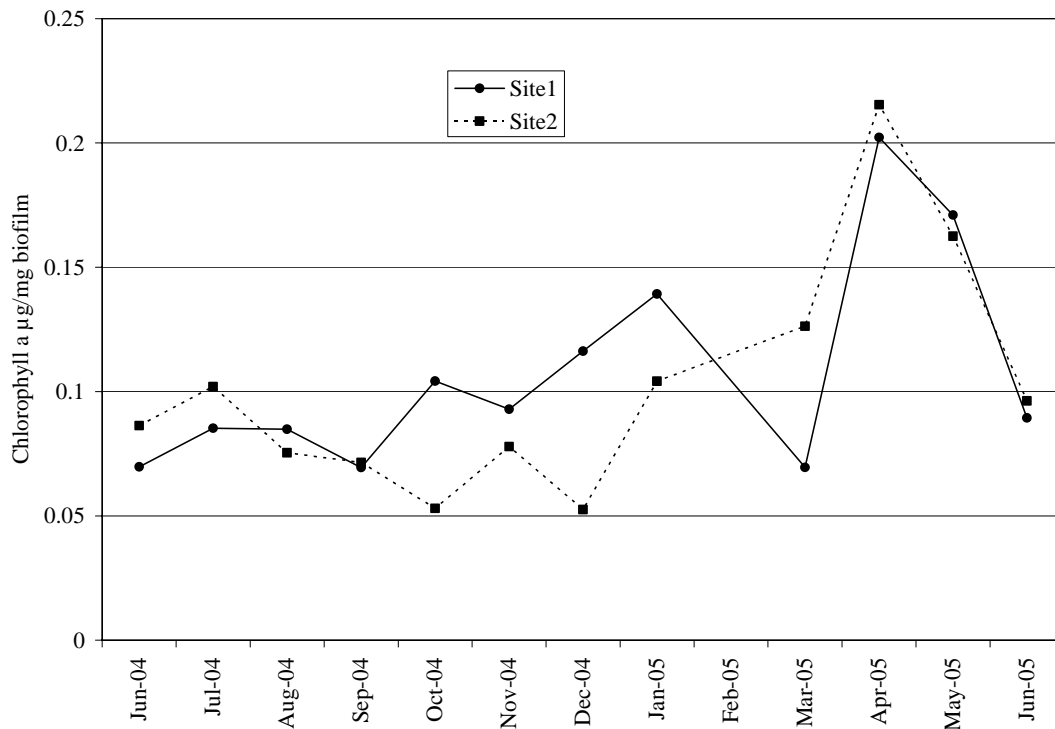


Fig. 3 Seasonal changes in chlorophyll *a* concentrations of biofilms collected during June 2004 – June 2005 from the littoral zone of Lake Constance. X-axis represents time in months, starting from June 2004 to June 2005 and Y-axis shows chlorophyll *a* content in  $\mu\text{g}/\text{mg}$  of biofilm

EPS content in pore water (i.e. cEPS, soluble EPS or colloidal EPS) ranged from 0.7-3.5  $\mu\text{g}/\text{mg}$  biofilm whereas; the other two extracts bEPS (bound EPS) and eEPS (EDTA extractable EPS) (0.12-0.93 and 0.21-0.98  $\mu\text{g}/\text{mg}$  of biofilm respectively) showed a comparatively low EPS. The temporal changes were observed in the EPS concentration of the biofilms collected at two different sites (Fig. 4). Site-1 had a high amount of EPS in Jun 04 then a gradual reduction until Jan 05 and from Mar 05 to Jun 05, again increase was observed (Fig. 4A). In contrast, Site-2 first showed a decrease in concentration until Sept 04, then a sudden increase in Jan 05 and Mar 05 (Fig. 4B). bEPS content and eEPS showed similar fluctuations in EPS concentration. At Site-1, bEPS and eEPS were higher in Nov 04 and May 05, whereas at Site-2 those were higher in Oct 04 then from Apr 05 onward (Fig 4A & B). Chlorophyll *a* was strongly correlated with carbohydrates content of cEPS (0.79) and with bEPS (0.75). It showed a moderate correlation with eEPS (0.45).

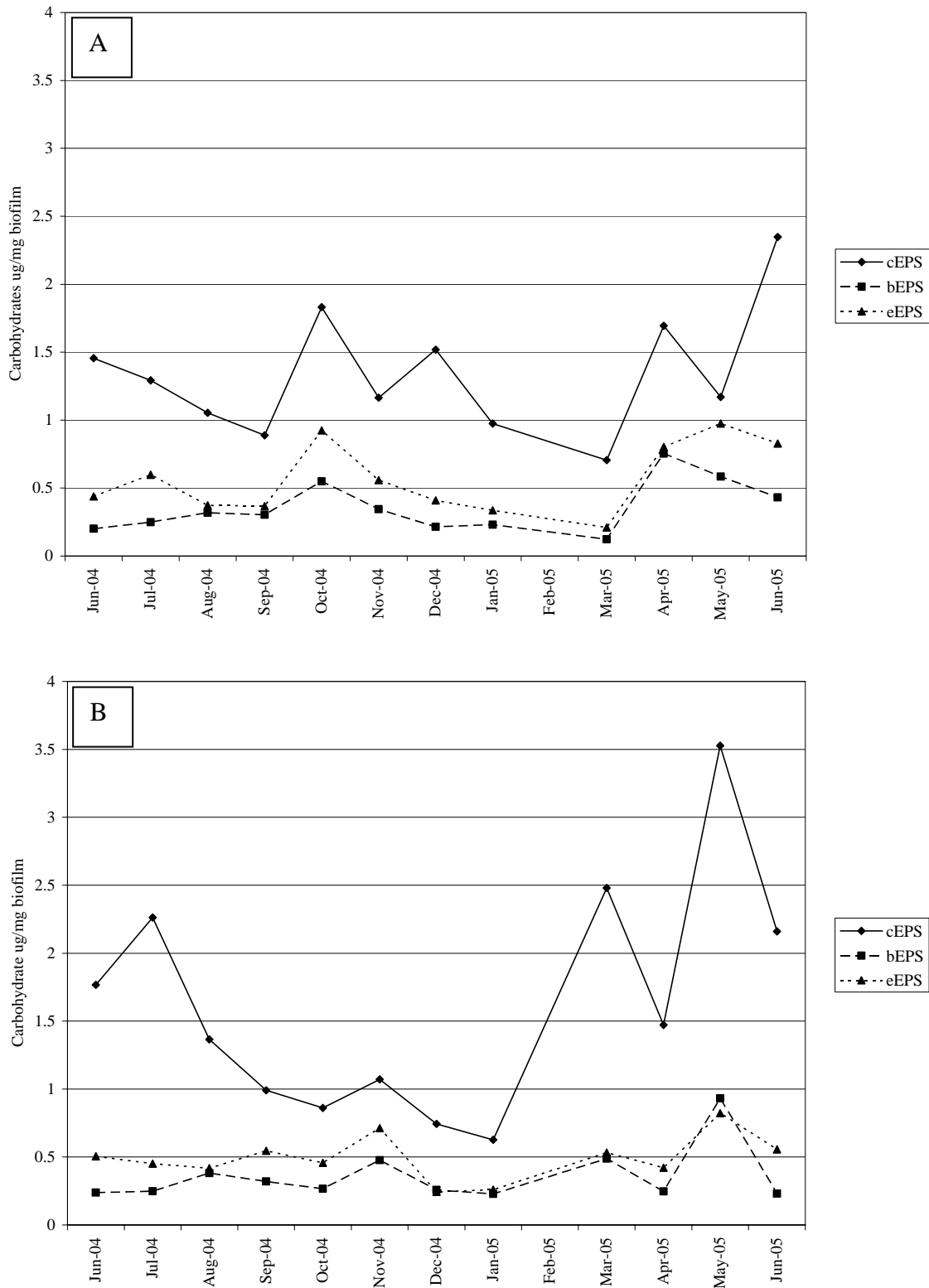


Fig. 4 Seasonal dynamics of three fractions of EPS with respect to site A Site-1 and B Site-2 from epilithic biofilm collected during Jun 2004 – Jun 2005. cEPS, soluble EPS / pore water EPS; bEPS, bound EPS; eEPS, EDTA soluble EPS. X-axis represents time in months, starting from June 2004 to June 2005 and Y-axis shows carbohydrate content in  $\mu\text{g}/\text{mg}$  of biofilm

### Discussion

Here we studied the seasonal dynamics of diatoms from the littoral zone of Lake Constance. Our study revealed the dominance of *Denticula tenuis*, *Achnanthes minutissima* and *Cymbella microcephala*. These taxa were recorded in nearly all samples throughout the year with only a difference in the abundance per month and per site. Only a few species contributed to more than 1 % of the total frustule count, other were either observed occasionally or represented by few frustules.

*D. tenuis* was the most dominant species in our study. This species is common in the lakes of Alps (Poulícková et al., 2004) and in smaller lakes of Alaska (Marciniak, 1986) and was among dominant species of epilithic biofilms from streams of Texas (USA) (Sherwood & Sheath, 1999) and Lake Saanajärvi (Finland) (Rautio et al., 2000). *D. tenuis* was also reported from limestone springs of Spain (Sabater, 1990) and from limestone lakes in Canada (Werner & Köhler, 2005) it is an indicator species for warmer water condition (Loseva, 1983), accordingly in our study it was dominant mainly during summer.

*A. minutissima* is a cosmopolitan diatom species and may deal with a broad spectrum of ecological conditions (Barbiero, 2000). It is very competitive species because of its small cell size and fast multiplication rate. It is also capable to attach strongly to the substratum (Barbiero, 2000). It is considered as one of the pioneer species that colonize on submerged surfaces and also was reported as a dominant species on various sites prone to disturbances. It was reported to be a dominant species in Lake Velencei, due to disturbance caused by high human activities (Ács et al., 2003). During our studies on the effects of water level fluctuations in the Lake Constance, *A. minutissima* was one of the dominant species and was established in mature biofilms. It was recorded throughout the year and was dominant during Jul 04 at both sites. (Barbiero, 2000) reported that *A. minutissima* was more abundant in summer than winter. In accordance with this, we observed that it was dominant during summer (mainly during Jul 04) and a gradual decrease in frustule counts was noted during winter at Site-2.

A multi-lake comparison showed that during autumn *A. minutissima* was replaced by *C. microcephala* (Barbiero, 2000). Similarly, in our study *C. microcephala* was dominant during the autumn i.e. Sept 04 and Nov 04 and then was replaced by *A. minutissima*.

Colonies of *Fragilaria* form long chains that do not attach firmly to the surface. *Fragilaria* species are also susceptible to displacement and colonies can easily be broken and suspended by the water currents. They also were demonstrated to have a faster recovery than most other diatoms (Peterson et al., 1990). Because of this peculiar type of dispersal mechanism, some colonies might be present in the open water, which may easily settle on the surfaces. This might explain the presence of a consistent number of *Fragilaria* species throughout the year. *Amphora inariensis* was found to be a successful species establishing during peak winter and then maintaining at a nearly constant number. Similar to *Fragilaria*, *Navicula cryptocephala* was present almost throughout the year.

When compared across various lakes, Stevenson et al. (1996) noted 17 most dominant taxa in littoral zones. Among them, seven were identified in an epilithic biofilm samples throughout the year, including *A. minutissima*, *C. microcephala*, *C. minuta*, *D. tenuis*, *Diatoma vulgare* and *Gomphonema olivaceum* *Fragilaria capucina* (Chapter 2).

Water levels are one of the important environmental factors, which highly influence the littoral zone. When water level recede biofilms from shallow areas are exposed to fluctuating temperature and can lead to desiccation. Increasing water levels make new surface available for colonization by the microbenthic communities. Species like *A. minutissima*, *C. microcephala* are among pioneer species in biofilm formation (Barbiero, 2000) and as water level increases in summer, these species start to colonize on the newly submerged surfaces. In summary, a correlation between various species and water levels suggests that WLF are one of the major environmental factors influencing littoral zone biofilms.

Seasonal changes in the temperature also affect the diversity of freshwater benthic communities (Stevenson et al., 1996). As Lake Constance is a prealpine lake, the epilittoral zone is exposed to high temperatures during summer and nearly freezing conditions during winter (Mörthl, 2003). At lower temperatures a reduction in overall diversity has been reported earlier (Stevenson et al., 1996) which was not observed in our study.

PCA analyses revealed clear seasonal dynamics and successional patterns at both sites. Even though there were differences with respect to abundance and species richness, they showed a relatively similar pattern of dominance and succession at both sites.

Chlorophyll *a* concentrations were used as a measure of biomass for photosynthetic organism in the biofilm and showed time dependant differences. High chlorophyll *a* concentrations were observed in biofilms collected in Apr 05. In this month, also a high richness of diatom species was observed. The temperature increases in spring, and nutrient level increase in winter due to the yearly water mixing. Water levels also increase because of melting of ice from Swiss Alps. All these reasons might contribute to proliferation or establishment of biofilms and higher diatom growth reflected by higher chlorophyll *a* content.

Diatoms are known for high amount of EPS production (Hoagland et al., 1993; Underwood & Paterson, 1993; Underwood & Paterson, 2003). In diatom dominated biofilms cEPS content showed a very high correlation with chlorophyll *a* (Staats et al., 2001; Stal, 2003; Underwood & Paterson, 1993; Underwood & Paterson, 2003), whereas this was not observed in biofilms dominated by cyanobacteria (Bellinger et al., 2005). cEPS can be extracted from biofilm / sediment just by centrifugation which separates the pore water and the biofilm. Extractions of bEPS are possible with the help of resuspension with water at 30 °C for an hour. This process releases EPS close to cells/sediment without cell leakage (de Winder et al., 1999). Biofilm contain high amounts of EPS, which is firmly attached to the gravel. Incubation of biofilm in the chelating agent such as 0.1 M EDTA for 16 h at 20 °C has been an accepted method (de Brouwer et al., 2002; de Winder et al., 1999; Stal, 2003). This method is also useful to fractionate EPS without cell lyses or less contaminations by intracellular carbohydrates (de Winder et al., 1999). Seasonal variation in the EPS fraction were related to the increase in microphytobenthos in summer and a reduction in the winter because of lower diatom biomass has been observed in intertidal mudflats (Staats et al., 2001). In accordance with this data, in our study Site-1 (all three types) and Site-2 (eEPS) showed high levels of EPS only during summer and low levels during winter. However, Site-2 showed large fluctuations in the cEPS content throughout the year.

At both sites, eEPS concentration was slightly higher than the bEPS. Where correlation between water extractable EPS and chlorophyll *a* was higher than eEPS. Correlation between eEPS and chlorophyll *a* showed that both were might not linked to each other.

In conclusion, a high diatom diversity estimated by Shannon's indices and PCA revealed a clear pattern of seasonal succession of diatoms in epilithic biofilms of Lake Constance has been observed. Chlorophyll *a* content and EPS showed high

correlation with each other. The EPS content was higher in summer as increasing water level and high temperature as well as availability of nutrients can boost diatom growth.

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## Chapter 4

### Isolation, cultivation, identification and phylogenetic analysis of diatoms from epilithic biofilms of Lake Constance

Rahul Bahulikar<sup>1</sup>, Linda Medlin<sup>2</sup> Kurt Mendgen<sup>1</sup> and Peter Kroth<sup>1</sup>

1. Faculty of Biology, University of Konstanz, University str. 10, Konstanz Germany
2. Alfred-Wegener-Institute, Am Handelshafen 12, Bremerhaven, Germany

Key words: Diatom cultivation, phylogeny, benthic diatoms

### **Abstract**

More than 100 uni-algal diatom strains were isolated and cultivated from epilithic biofilms of Lake Constance, most of them pennate diatoms. They comprised of 44 different species and belonged to 20 different genera. The strains were isolated from biofilms throughout the year over a period of two years to cover a major part of the diatom diversity observed during the year. The cultivated diatoms were identified and characterized on the basis of morphological traits as well as by sequencing the 18S rDNA region was used as a basis for phylogenetic analysis. We observed that in some cases the morphological and phylogenetical characters yielded similar results while some of the morphological characters similar strains did not group together in the phylogenetic tree.

## **Introduction**

Diatoms are the most dominant group of eukaryotic organisms in various water bodies and are represented by more than 10,000 described species (Mann, 1999). They are ubiquitous and can be found in all types of environments such as oceans, seas, lakes, rivers, streams, moist places and wet lands and in unusual places like whale skin, hot springs, etc (Mann, 1999). Diatoms have also been reported to be present as endosymbionts in dinoflagellates (Chesnick et al., 1997). In freshwater and marine environments they may be present as either free living (planktonic) or attached (benthic).

Diatoms are ecologically important organisms. In oceans about 40% of the fixed CO<sub>2</sub> is by diatoms (Mann, 1999). In shallow lakes, the productivity of the benthic diatoms can be compared with that of planktonic diatoms (Wetzel, 1964). Therefore, benthic areas are also considered as important habitats for primary production.

The presence of silicious rigid cell wall (frustule) is one of the characteristic features of diatoms. Diatoms are diploid organisms and during mitotic cell division, new cell walls are formed within the epi- and hypo-theca of the parent cell. One of the daughter cells has the same size as the parent whereas the second daughter cell is relatively smaller. After many such cell divisions, the overall size of the cells in the population may decrease to a critical level (Round, 1982). Then cells undergo a sexual reproduction to restore maximum cell size (Kooistra et al., 2003; Round, 1982).

Diatoms are classified morphologically based on their ornamentation of the frustules. This ornamentation is also well preserved in fossil diatoms, which can be easily compared with the modern diatoms. Diatoms are considered to have evolved from spherical monads or from cyst-like forms of chrysophytic algae (Medlin & Kaczmarska, 2004). Diatoms are mainly divided into two groups: centric and pennate. Pennates are further divided into raphid and araphid diatoms which are separated from centrics and each other by a number of different characters such as symmetry of the frustules, presence or absence of raphe, etc. (Medlin & Kaczmarska, 2004).

Recent phylogenies based on the 18S rDNA sequence comparisons revealed a monophyletic origin of diatoms within the pigmented heterokont lineage (Sims et al., 2006). Furthermore, diatoms are related to Bolidophyceae (Guillou et al., 1999),

which are picoplanktonic algae with a simple cellular organization. Morphological separation of diatoms into centrics and pennates which is not supported by the molecular approach (Medlin et al., 1993). Paraphyletic origin of araphid pennates has been supported by various molecular markers such as RbcL, LSU, Tuf A (Medlin et al., 2000; Medlin et al., 1996) and cox I (Ehara et al., 2000).

Diatoms are known to be important members of epilithic biofilms (Barbiero, 2000; Hawes & Smith, 1994; King et al., 2000; Sherwood & Sheath, 1999; Soininen, 2002). In order to study the role of diatoms in epilithic biofilms our primary aim was to

1. Isolate and cultivate various diatoms from epilithic biofilms of Lake Constance
2. Identified the isolates using the classical morphology
3. Used a molecular approach where 18S rDNA sequence homology based phylogenetic trees of the cultivated diatoms were constructed and related with other diatom sequences.

## **Materials and Methods**

### *Isolation and cultivation of diatoms*

Epilithic biofilms from littoral zone of Lake Constance (Germany, 47°41'N, 9°11'E) were collected at various seasons. In the laboratory, small amounts of biofilms were scraped off and were diluted using sterile diatom medium (DM) (Watanabe, 2005). Then either single live diatom cells were picked using an inverted microscope with a lab made micromanipulator and inoculated 24 well microtitre plates containing 1 ml of sterile DM medium. In addition, 50 µl of diluted biofilms were spread on DM plates (DM + 12.5 g/l agar). The resultant cultures were maintained at 16 °C for 16 h, 50 µE illumination provided by cool-white fluorescent tubes. Plating of biofilm algae on Petri-dishes resulted in growth of small colonies within 8-10 days and many colonies of diatoms were picked using toothpick and inoculated in 24 well plates containing 1 ml of DM. After a growth period of 2-3 weeks, the cultures were observed microscopically to identify of single diatom morpho-types. Wells containing more than one diatom isolates were again screened until unialgal cultures were obtained. All cultures were numbered accordingly and were incubated at same light intensity, temperature and on the fresh DM.

### *Microscopy and morphological identification*

All cultures were checked microscopically for purity (i.e. presence of only single diatom species in a culture) before cleaning their frustules. Samples for diatom identification were incubated at 95 °C for 3 hrs in 10% H<sub>2</sub>O<sub>2</sub>, followed by over night incubation in 10% HCl at room temperature to remove carbonates (Battarbee, 1986; Battarbee et al., 1999). To remove the residual HCl samples were washed twice with distilled water. Cleaned samples were suspended in distilled water and mounted in Naphrax (Euromex Microscopes, Netherlands) and numbered accordingly. Cleaned diatom frustules were observed either using a light microscope or a scanning electron microscope. Light microscopy was done at 1000X magnification using an Olympus microscope (BX51) equipped with a Nikon digital camera (DMX-1200). For SEM examination, a drop of water containing cleaned frustules was placed on aluminum stubs, coated with gold, and observed under scanning electron microscope (model 505 Philips NV, the Netherlands). Diatom cells were identified to the highest possible taxonomic resolution. All diatoms were identified based on the key for morphological

characters provided by Krammer & Lange-Bertalot (1986-1991). Most araphid diatoms were identified based on the distinguishing characters mentioned in Table 1.

#### *DNA extraction and quantification*

Around 40-50 mg of cell pellets was used for extracting DNA. A modified CTAB method (Murray & Thompson, 1980) was used and yielded good quality of the DNA. Resultant dried pellets of the DNA were dissolved in 50  $\mu$ l of TE buffer (10 mM Tris/Cl, pH 8.0, 1 mM EDTA). DNA of each samples were examined for its quality and quantified by agarose gel electrophoresis against known standards and also spectrophotometrically and were diluted to a concentration of 10 ng/ $\mu$ l.

#### *PCR and sequencing*

The 18S rDNA fragment were PCR amplified in 25  $\mu$ l reaction containing 10-20 ng of diatom genomic DNA, 1 mM dNTPs, 0.5  $\mu$ M of forward primer (AAC CTG GTT GAT CCT GCC AGT) and 0.5  $\mu$ M of reverse primer (TTG ATC CTT CTG CAG GTT CAC CTA C), 1X Eppendorf PCR reaction buffer and 1 unit of DNA polymerase (Eppendorf, Germany). PCR steps were included initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 53 °C for 40 s and 72 °C for 2 min, and final extension at 72 °C for 10 min. Quantity and length of the PCR product was conformed using agarose gel electrophoresis against known standards. PCR product was clone in to pGMT (Promega, Germany) and resulting plasmid was sequenced using SP6, T7 and 892 forward and reverse primers either using ABI sequencer according to manufacturer's instructions or at GATC biotech (Konstanz, Germany). Primer sequences were obtained from (Medlin et al., 1988).

#### *Data analysis*

Forward and reverse sequences were combined in Seqman program from DNASTAR software (DNASTAR, USA) and were compared with the diatom sequences available in GenBank (<http://www.ncbi.nlm.nih.gov/>). All sequences were added to ARB (Ludwig & Strunk, 1996; Ludwig et al., 2004). Published sequences of diatom with were downloaded from the GenBank and aligned using Fast-aligner tool of the ARB. A 18S rDNA database was prepared of >200 sequences (55 from our isolates and remaining were from the GenBank). Phylogenetic trees were constructed using neighbor-joining method as implemented in ARB.

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Table 1 Differentiating characters of the members from family Fragilariaceae

Genus	Areolar	Lp	number of Lp	pore fields	Pl	Girdle band	sterum	spines	old name / type species
<i>Fragilaria</i>	simple pores	+	one	large, poroid	+	uniseriate	narrow	between areolae	<i>F. capucina</i>
<i>Staurosira</i>	simple pores	-	-	tiny, few pores	-	plain	narrow to broad	between areolae	<i>F. construens</i>
<i>Staurosirella</i>	linear bars	-	-	large, poroid	+	plain	wide	between areolae	<i>F. pinnata, F. lapponica</i>
<i>Stauroforma</i>	simple pores	-	-	absent or small pores	-		very narrow or absent	between areolae branched at tip	<i>F.</i>
<i>Pseudostaurosira</i>	simple pores volae	-	-	absent or small pores	+	plain	wide	between areolae	<i>F. brevistriata</i>
<i>Pseudostauropsis</i>	2-6 areola per stria	-	-	absent or small pores	+	plain	wide	on areolae	<i>P. connecticutensis</i> no <i>Fragilaria</i>
<i>Punctastriata</i>	simple pores	-	-	one small pores	+	plain	wide	on and between areolae	<i>P. kinearis</i> no <i>Fragilaria</i> as type
<i>Neofragilaria</i> = <i>Fragilariopforma</i>	Broad mutliporoid	+	one	small poroid	+	uniseriate	very narrow or absent	between areolae	<i>Fragilar virescens</i>
<i>Fossula</i>	simple pores	+	one	striae radiate around apex	-	multiseriate	narrow	-	<i>Fragilaria arctica</i>
<i>Ulnaria</i>	pore with rotae	+	one	recessed poroid with rim	-	uniseriate	narrow	-	<i>Synedra ulna</i>
<i>Tabularia</i>	multiseriate alveolate	+	two, rarely one	recessed poroid with rim	-	plain	narrow to broad	-	<i>Synedra fasciculata</i>
<i>Nanofrustulum</i>	simple pores volae	-	-	one ringed pore	-	plain	narrow to broad	between areolae	<i>Fragilaria shiloi</i>

Lp, Labiate process; Pl, Plaque; Courtesy Linda Medlin (AWI Bremerhaven, Germany)

## Results

### Cultivation

More than 100 unialgal diatom cultures were established by spreading and/or micromanipulation. They represented a total of 44 species and 20 genera of diatoms from epilithic biofilms of the littoral zone of Lake Constance. The genus *Cymbella* contributed for seven species, whereas *Navicula* was represented by five species (Table 2).

Table 2 List of diatom species isolated and cultured from epilithic biofilms of Lake Constance

No	Species name	Isolate no(s).
1.	<i>Achnanthes minutissima</i> Kützing	A-03, B-06, D-19, D-37, D-38, I-117 etc.
2.	<i>Cymbella caespitosa</i> (Kützing) Brun	D-52
3.	<i>Cymbella microcephala</i> Grunow	B-04, B-08, D-23, I-04, I-26
4.	<i>Cymbella lanceolata</i> (Ehrenburg) Kirchner	D-55
5.	<i>Cymbella minuta</i> Hilse	B-07, I-35, I-51, I-147
6.	<i>Cymbella cistula</i> (Ehrenburg) Kirchner	D-143
7.	<i>Cymbella silesiaca</i> Bleisch	D-41, D-42, D-133
8.	<i>Cymbella vulgata</i>	I-34
9.	<i>Diatom tenuis</i> Agardh	D-45
10.	<i>Diatoma vulgaris</i> Bory	D-119
11.	<i>Diatoma vulgaris</i> var. <i>brevis</i> Grunow	D-21, D-22, D-117
12.	<i>Diatoma vulgaris</i> var. <i>linearis</i> Grunow	D-141, D-144, D-147
13.	<i>Gomphonema truncatum</i> Ehrenberg	D-124
14.	<i>Gomphonema olivaceum</i> (Hornemann) Brébisson	D-80, D-140
15.	<i>Gomphonema</i> cf. <i>clavatum</i>	D-87
16.	<i>Gomphonema parvulum</i> (Kützing) Kützing	D-90
17.	<i>Navicula cincta</i> (Ehrenberg) Ralfs	D-64
18.	<i>Navicula capitatoradiata</i> Germain	D-92
19.	<i>Navicula radiosa</i> Kützing	D-72
20.	<i>Navicula cryptocephala</i> Kützing	D-94

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21.	<i>Navicula cf. cryptotenella</i> Lange-Bertalot	D-102
22.	<i>Nitzschia palea var. debilis</i>	D-25
23.	<i>Nitzschia angustata</i> Grunow	D-69
24.	<i>Nitzschia palea</i> (Kützing) W. Smith	D-95, D-96
25.	<i>Nitzschia dissipata</i> (Kützing) Grunow	D-99
26.	<i>Nitzschia peisonis</i> Pantocsek	D-104
27.	<i>Nitzschia palea var. muja</i>	D-139
28.	<i>Synedra angustissima</i> Grunow	D-16
29.	<i>Synedra ulna</i> (Nitzsch) Ehrenberg	D-32, D-33, D-145
30.	<i>Synedra vaucheria</i> (Kützing) Kützing	D-109
31.	<i>Fragilaria capucina</i> Desmazères	D-06, A-06
32.	<i>Pseudostauroopsis</i>	Dx-7, D-91, D-48, I-01, F-02, F-05, F-03, D-79, I-23, I-61
33.	<i>Staurosira</i> Ehrenberg	C-05, D-20, I-141,
34.	<i>Punctastriata</i>	D-35, C-07, D-05, E-05
35.	<i>Pinnularia viridis</i> (Nitzsch) Ehrenberg	D-11, D-12
36.	<i>Nanofrustulum</i>	D-07,
37.	<i>Fragilaria</i> species	D-121, D-108
38.	<i>Cymatopleura solea</i> (Brébisson) W. Smith	D-14
39.	<i>Cymatopleura apiculata</i> W. Smith	D-138
40.	<i>Caloneis alpestris</i> (Grunow) Cleve	D-62
41.	<i>Caloneis silicula</i> (Ehrenberg) Cleve	D-13
42.	<i>Amphora ovalis</i> (Kützing) Kützing	D-04
43.	<i>Asterionella ralfsii</i> W. Smith	D-44
44.	<i>Surirella brebissoni</i> Krammar & Lange-Bertalot	D-49
45.	<i>Stephadodiscus hatschii</i> Grunow	D-01
46.	<i>Cyclotella meneghiana</i> Kützing	D-02
47.	<i>Melosira variance</i> Agardh	D-29, D-30

Spreading of diluted biofilm on agar plates was found to be useful to cultivate species like *Achnanthes minutissima*, *Cymbella minuta*, *C. microcephala* and various chain-forming diatoms of the family Fragilariaceae, whereas micromanipulation

techniques were useful for the isolation of most of the large sized diatoms like *Synedra*, *Pinnularia* and *Cymbella* (Table 1).

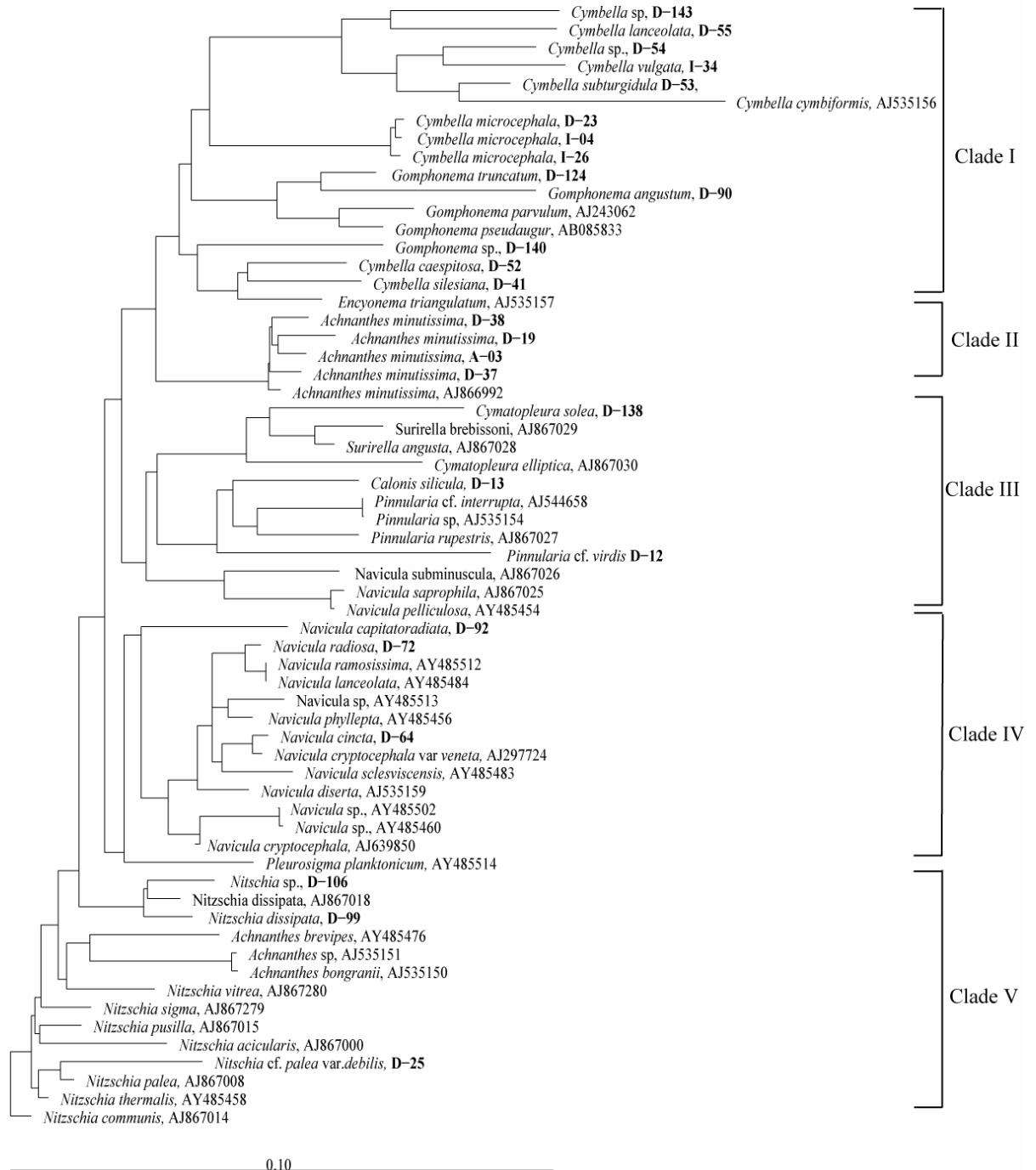


Fig. 1 Phylogenetic tree of raphid diatoms constructed using neighbor joining parameter of ARB software based on 18S rDNA sequences. Taxa with accession number were from the GenBank and taxa with isolate numbers are from epilithic biofilms from Lake Constance. Tree is showing presence of five clades.

In our isolates, only 4 centric diatoms were found, namely *Melosira variance*, *Stephanodiscus* sp, *Cyclotella* sp., whereas all other species belonged to the Pennales (Table 1). The order Pennales is further divided in to two suborders, the Araphidineae and the Raphidineae.

### *Raphids*

We identified raphid diatoms that belonged mainly to the four families Achnanthaceae (*A. minutissima*), Naviculaceae (*Amphora*, *Caloneis*, *Cymbella*, *Gomphonema*, *Navicula* and *Pinnularia*), Bacillariaceae (*Nitzschia*) and Surirellaceae (*Cymatopleura*, *Surirella*). A neighbor joining phylogenetic tree revealed the presence of five main clades (Fig. 1)

Clade I consists of diatoms of the family Naviculaceae such as *Cymbella* and *Gomphonema*. *Cymbella* is divided into three different clusters of which the first cluster contains various isolates of *Cymbella* from our study with *C. cymbiformis*. The second cluster is dominated by *C. microcephala* but unfortunately no *C. microcephala* related sequences are available in the GenBank. The third cluster is represented by species belonging to the subgenus *Encyonema*. All *Gomphonema* sequences from this analysis are grouped in a single cluster.

Clade II contains Achnanthaceae, which contain all isolated diatoms from *A. minutissima*. In Clade III, two families Surirellaceae (*Cymatopleura* and *Surirella*) and Naviculaceae (*Pinnularia*, *Calonis* and *Navicula*) clustered together. Clade IV represents all diatoms of the genus *Navicula* and one diatom of the genus *Pleurosigma*. Clade V represents the two families Achnanthaceae and Bacillariaceae group. *Nitzschia* group, *N. dissipata* and other *Nitzschia* species were clearly separated from the three species of *Achnanthes*.

### *Araphids*

The genus *Fragilaria* has been recently revised into various genera on the basis of morphological characters (Williams, 2006; Williams & Round, 1987). The distinguishing characters of these genera are listed in Table 2 and served for the identification of our isolates from this group (Fig. 2). Classifications schemes previously proposed by (Williams & Round, 1986; Williams & Round, 1987) and (Krammer & Lange-Bertalot, 2000) are shown in Table 3. *Fragilaria capucina* is characterized by the presence of

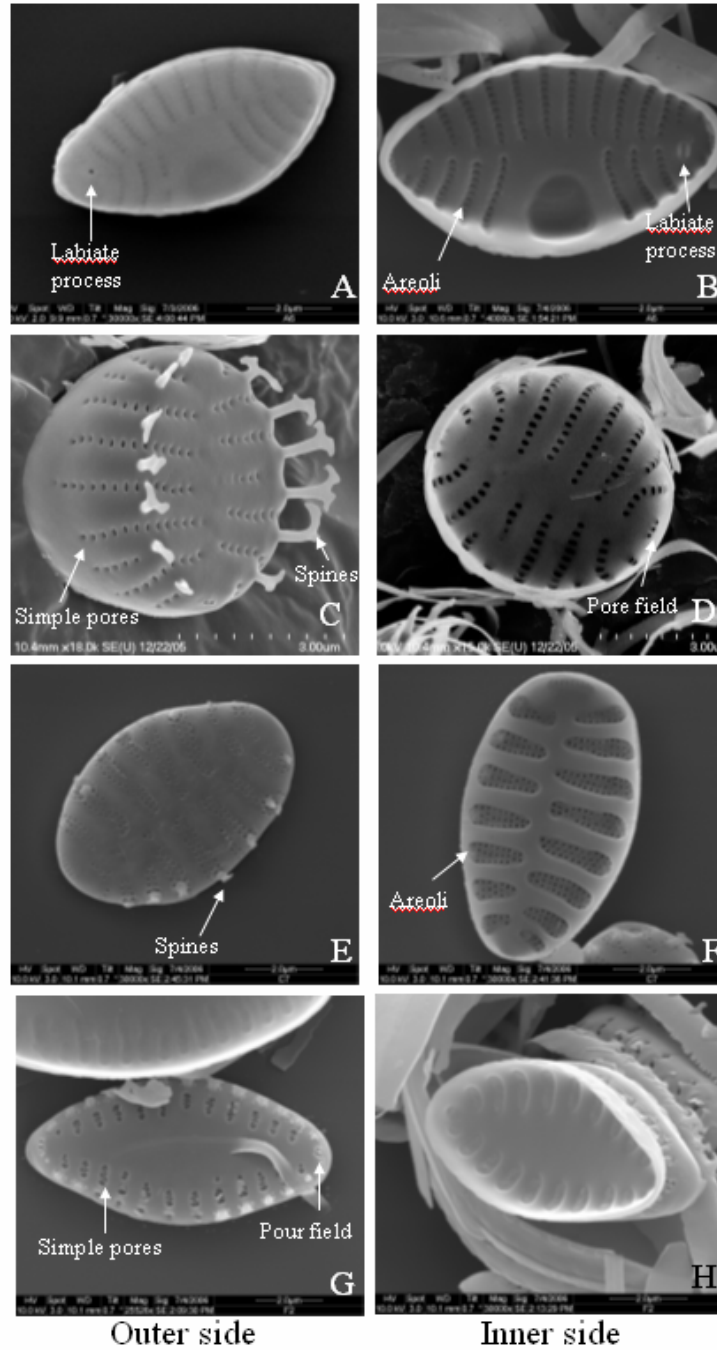


Fig. 2 Electron micrographs are showing distinguishing characters of frustules of four genera from Fragilariaceae A. *Fragilaria capucina* outer view B. inside view of the same diatom showing presence of labiate process and uniseriate nature of areoli C and D. Outside and inside view of *Staurosira* showing simple pores, spines alternate to it and the presence of few tiny pores in the pore field, E and F outside and inside view of *Punctastriata* demonstrating spines on and alternate to aerioli, G and H *Pseudostauropsis* illustrating outside and inside view showing complex nature of pores, spines on the areoli and small pore fields

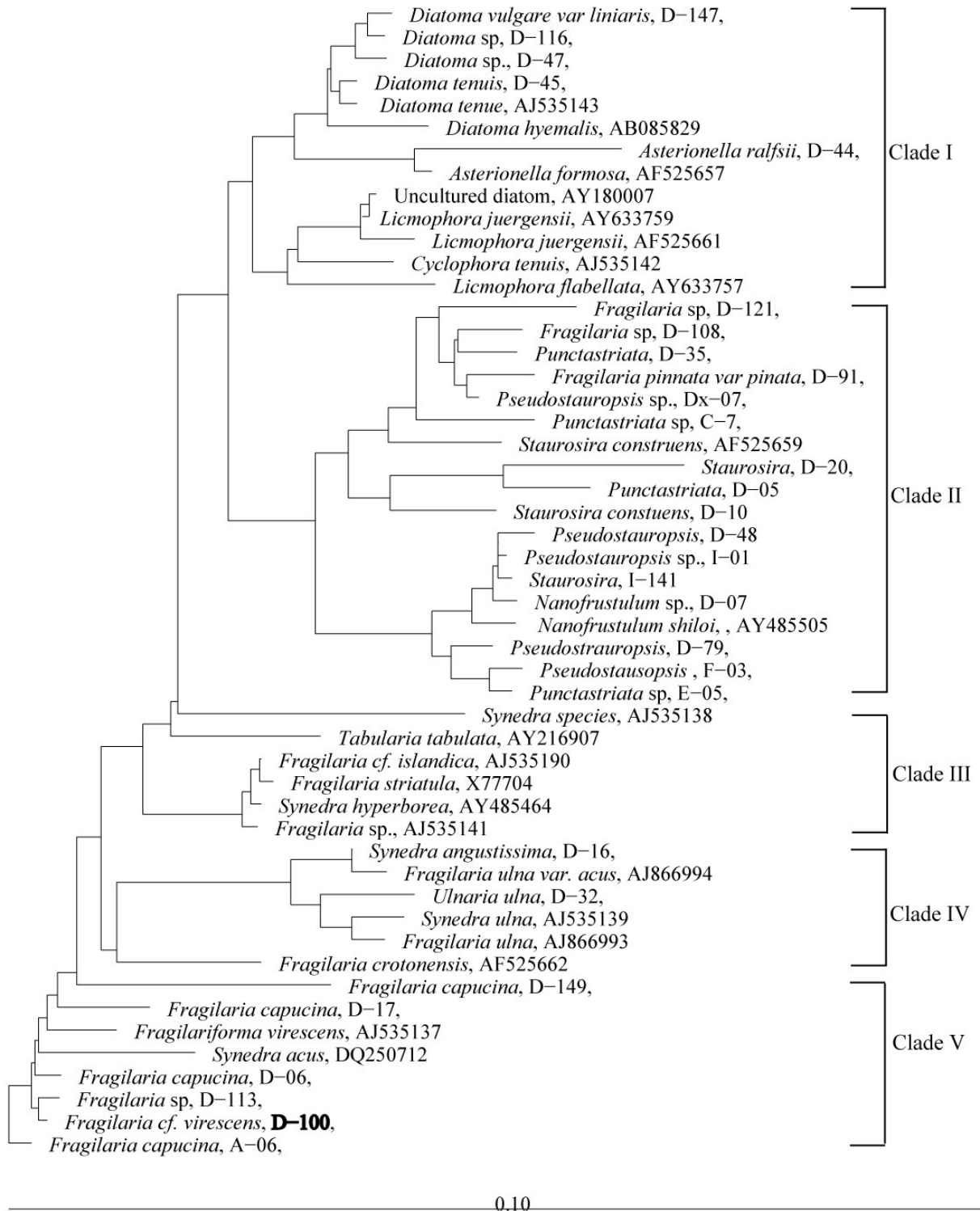


Fig. 3 Phylogenetic tree of araphid diatoms constructed using neighbor joining parameter of ARB software based on 18S rDNA sequences. Taxa with accession number were from the GenBank and taxa with isolate numbers are from epilithic biofilms from Lake Constance. Tree is showing presence of five clades

simple pores, a labiate process, the spines on the areolae and a large polar field (Fig 2 A and B). However, labiate processes were absent in *Pseudostauroopsis*, *Staurosira*, *Punctastriata* etc. (Table 2). Latter three genera are differentiated based on the structure of areolae. In *Staurosira*, areolae are made of simple pores (Fig, 2C and D Table 2), whereas it is broad multipoloid in *Punctastriata* (Fig. 2 E and F). In *Pseudostauroopsis*, stria contains 2-6 areola. The spines in these genera are present between the areolae (Fig. 2G and H).

The phylogenetic tree based on 18S rDNA of araphid diatoms is divided into 5 different clades (Fig. 3). With respect to the classification of (Williams, 2006) (Table 3), Clade I showed genus-wise clustering of *Diatoma* and *Asterionella*. Clade II represents diatom genera *Pseudostauroopsis*, *Nanofrustulum*, *Staurosira* and *Punctastriata*. Classification of these genera up to now is mainly based on the morphology. Moreover very few 18S rDNA sequences of the above mentioned genera are available in the database. In this study, various isolates that were identified as a particular genus on the basis of morphology did not group together in the phylogenetic tree. Thus, a very high heterogeneity was observed in the phylogeny even though the diatoms of same genera were morphologically similar. Clade III comprised of some species from the genera *Tabularia*, *Synedra* and *Fragilaria*. Clade IV consists of diatoms from the genus *Synedra* (new name *Ulnaria*). Clade V showed the presence of *Fragilariforma*, *Fragilaria* and *Synedra*.

## Discussion

### *Cultivation*

Benthic biofilms from lentic environments are usually dominated by diatoms (Stevenson et al., 1996) and Lake Constance was also not an exception. The 18S rDNA clone library approach was employed to unravel the eukaryotic diversity from epilithic biofilms of Lake Constance and showed highest abundance of diatom specific sequences compared to the other algal groups (Bahulikar & Kroth unpublished data). We further investigated the community structure of diatoms from epilithic biofilms (Chapters 2 and 3) by a classical method. In the present study, we cultivated various diatoms from the biofilm and analyzed their phylogeny of various diatoms from epilithic biofilms of Lake Constance.

Two different techniques were used for the cultivation of diatoms from epilithic biofilms. Cultures obtained by the spreading technique yielded diatoms of relatively smaller size (<15 µm) as compared to those obtained by micromanipulation method. Most of the cultures represented the dominant diatoms from our earlier reports (Chapters 2 and 3). A higher number of isolates were obtained by the spreading technique. These diatoms belonged to *A. minutissima*, *C. microcephala* or *Fragilaria* species. However, by manipulation, it was easier to pick single cells and this approach was useful for the isolation and cultivation of rare and / or diatoms of relatively larger size (>10 µm in length) e.g. *Cymatopleura solea*, *Pinnularia*, *Synedra*, *Cymbella* species etc.

### *Relating morphology, phylogenetic and extracellular polysaccharide structures*

The monophyletic origin of the diatoms based on 18S rDNA sequences has been demonstrated earlier (Medlin et al., 1993) and within diatoms centrics are paraphyletic, whereas pennates are of monophyletic origin (Kooistra et al., 2003). Further, the pennates showed several clades containing araphid pennates and a single clade represented by raphid pennates (Kooistra et al., 2003). The tree revealed a clear separation between araphid pennates and raphid pennates (data not shown).

In raphid pennates, clade I showed the presence of *Cymbella* and *Gomphonema* and the divergence of *Cymbella* related diatoms were highly supported by monoraphid taxa (Medlin & Kaczmarek, 2004). Morphologically *Cymbella* is divided into three

subgenera: *Encyonema*, *Cymbella* and *Cymbopleura* (Krammer & Lange-Bertalot, 1986-1991). In accordance, phylogenetic analysis showed a separate cluster for each subgenus. Our isolates belonged to all the three subgenera. The subgenus *Cymbella* contains mainly stalk producing diatoms e.g. *C. subturgidula* and *C. cistula* (Wustman et al., 1997) whereas, the other two subgenera showed capsules or tube like structures e.g. *C. microcephala* (*Cymbopleura*) and *C. caespitosa* (*Encyonema*) (Hoagland et al., 1993). Stalk production of *Gomphonema* from the clade I has been reported previously (Dawson, 1973; Hoagland et al., 1993; Huntsman & Sloneker, 1971)(Chapter 5).

Clade II contain various isolates of *A. minutissima* and they were also produces tubes like structures (Chapter 5). The isolates from Lake Constance from other clades showed no tube or stalk like EPS structures.

Araphid pennates obtained from epilithic biofilms of Lake Constance were from a single family the Fragilariaceae. In these diatoms, cell-cell / cell-substratum attachment is mediated by EPS pads. Species of *Asterionella* and *Diatoma* are phylogenetically related to each other and in both genera, cell-cell attachment is restricted to a small point on the frustule. In *Asterionella*, (mostly) star shaped colonies are formed due to a small globular EPS pads secreted on both wall faces, whereas in *Diatoma*, characteristic zigzag colonies are a result of pads secreted on opposite wall faces (Hoagland et al., 1993). Such apical pads also help *D. vulgaris* to attach surfaces firmly (Ács, 1998; Hoagland et al., 1993).

Clade II in the araphid tree contain ribbon like colony forming diatoms such as *Pseudostauroopsis*, *Punctastriata*, *Staurosira* and *Nanofrustulum* where intercellular pads are responsible for maintaining colony integrity. As these colonies easily break and may be suspended by water currents, which might helps in dispersal (Peterson et al., 1990). Similar colony morphology was observed in morphologically and phylogenetically distinct taxa.

In *Synedra* species, a monophyletic group was observed in the phylogenetic tree. This group is characterized by basal pads which are responsible for cell-substratum attachment and which are 5-10 times stronger than stalks (Hoagland et al., 1993). Therefore, the cells can remain attached to the substratum even in the strong water currents. As cells divide, characteristic rosette colonies develops (Hoagland et al., 1993).

Most of the *Fragilaria* are probably benthic, epiphytic algae and are considered as monophyletic genera (Sims et al., 2006). Classification based on morphology in *Fragilaria* might be useful has been a debated for long time (reviewed in (Williams, 2006) (Table 3).

Table -3 Revision of the Genus *Fragilaria*, reproduced from (Williams, 2006)

Williams and Round	Krammer & Lange-Bertalot
Family Node A	Family Node A
Sub-Family – Node B	Genus <i>Fragilaria</i> (including <i>Fragilariforma</i> )
Genus- <i>Fragilariforma</i>	Sub-Genus- <i>Fragilaria</i> *
Genus- <i>Diatoma</i> *	Sub-Genus- <i>Synedra</i> *
Genus- <i>Distrionella</i>	Sub-Genus- <i>Tabularia</i> *
Genus- <i>Tetracyclus</i>	Sub-Genus- <i>Ctenophora</i>
Genus- <i>Odontidium</i>	
Genus- <i>Oxyneis</i>	
Genus- <i>Tabellaria</i>	
Genus- <i>Asterionella</i> *	
Sub-Family – Node C	
Genus- <i>Fragilaria</i> *	
Genus- <i>Synedra</i> *	
Genus- <i>Catacombas</i>	
Genus- <i>Hyalosynedra</i>	
Genus- <i>Tabularia</i> *	
Genus- <i>Ctnophora</i>	
Genus- <i>Neosynedra</i>	
Sub-Family – Node D	
Genus- <i>Staurosira</i> *	
Genus- <i>Matyana</i>	
Genus- <i>Staurosirella</i>	
Genus- <i>Punctastriata</i> *	
Genus- <i>Pseudostaurosira</i>	

\* Genera were included in molecular analysis

Most of these genera were combined under *Fragilaria* by Krammer & Lange-Bertalot (2000). Recent revisions, were based on very distinct characters examined mainly by SEM (Williams, 2006; Williams & Round, 1987). Both systems of classification when compared with the molecular data, showing a correlation only at a node level as described by Krammer & Lange-Bertalot (2000). Except *Diatoma* and *Synedra*, none of the other species were grouped according to genera, although morphological characters are very distinct. This suggests a very high heterogeneity within the groups. When we compare the phylogeny of Fragilariaceae with the two systems of classification, the molecular analysis supports the system by Krammer & Lange-Bertalot (2000) compared to the system proposed by Williams & Round (1987).

Based on morphology and clustering of various genera in phylogenetic tree, family fragilariaceae show paraphyletic nature (Kooistra et al., 2003; Sims et al., 2006). In accordance with this, our study showed similar results.

In conclusion, we have cultivated a large number of diatoms from epilithic biofilms of Lake Constance mainly belonging to the pennates. With respect to morphological characters raphid and araphid groups clustered separately in the phylogenetic analysis. Furthermore, within groups no strict genera wise grouping was observed in most of the cases.

**Acknowledgements**

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## Chapter 5

# Localization of EPS components secreted by freshwater diatoms using differential staining with fluorophore-conjugated lectins and other fluorochromes

Rahul A. Bahulikar and P. G. Kroth\*

Fachbereich Biologie, Universitaet Konstanz, 78457 Konstanz, Germany

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### Abbreviations

CAE, cell wall associated EPS; DM, diatom medium; DAPI, 4',6-diamidino-2-phenylindole; DTAF, 5-(4,6-Dichloro-s-triazin-2-ylamino)fluorescein, EPS, extracellular polymeric substances; FITC, fluorescein isothiocyanate; Fuc, L-Fucose; Gal, Galactose; Glc, Glucose; GlcNAc, N-acetyl-D-Glucosamine; GalNAc, N-acetyl-D-Galactosamine; Man, Mannose;

## **Abstract**

Diatoms produce extracellular polymeric substances (EPS) which mainly consist of carbohydrates and which may form different morphological structures. We studied the localization and structure of EPS secreted by 17 diatom species that were isolated from epilithic biofilms from the littoral zone of Lake Constance (Germany). We used six different FITC-labelled lectins and DAPI to localize and visualize the structure of secreted EPS and cell wall associated EPS (CAE), while DTAF was useful to label CAE only. The diatoms were categorized according to the respective structure of the secreted EPS i.e. pads, capsules, tubes or stalks, etc. Among eight pad-producing diatoms, three *Fragilaria* species showed variable lectin binding indicating the presence of different carbohydrate components. Other pad forming diatoms like *Synedra*, *Diatoma*, *Asterionella*, and *Melosira* generally showed binding to at least two different lectins. On the other hand, we did not observe any lectin binding to the capsules of *Staurosira* and one *Achnanthes* isolate. We further detected differences in the carbohydrate composition of tube-like EPS structures in two *Cymbella* species and were able to demonstrate developmental stages of tube formation. The stalk secreting species *Gomphonema* and *Cymbella* showed labelling by only one lectin. On the other hand, in *Caloneis alpestris*, a highly complex nature of the EPS could be predicted as it stained by all used lectins and fluorochromes.

## Introduction

Pennate diatoms are often the dominant eukaryotic members of phototrophic biofilms and early colonizers of natural and artificial substrata (Wetherbee *et al.*, 1998). Diatoms possess the ability to attach to the substratum either temporarily or permanently. A temporary attachment includes characteristic diatom movements (gliding), whereas permanent attachment occurs when diatoms produce various extracellular structures. Gliding and extracellular translucent structure formations are mainly associated with the secretion of extracellular polymeric substances (EPS). Before gliding, pennate diatoms first attach to the substratum by the secreted EPS (Wetherbee *et al.*, 1998). Permanent attachment is achieved by continuous secretion of EPS, that may form morphological structures classified as adhering sheaths/capsules, tubes, pads or stalks (Hoagland *et al.*, 1993). Carbohydrates are the main components of EPS (Staats *et al.*, 1999; Stal & de Brouwer, 2003), and may also contain very small amounts of proteins (Staats *et al.*, 1999), glycoproteins (Lind *et al.*, 1997; Chiovitti *et al.*, 2003a) and uronic acids (Staats *et al.*, 1999; de Brouwer & Stal, 2002; Chiovitti *et al.*, 2003a, b). Many diatoms are able to secrete very large amounts of EPS in nature and in cultures, especially in the stationary phase (Staats *et al.*, 1999; de Brouwer & Stal, 2002; de Brouwer *et al.*, 2002). Most pennate diatoms secrete EPS through the longitudinal slit present in the silica wall known as the raphe or the apical pore field in the cell wall, while some centric diatoms use axillary pores (Hoagland *et al.*, 1993).

There are many reports on extracted EPS and its chemical composition, either from field samples (Yallop *et al.*, 2000; de Brouwer & Stal, 2001; Perkins *et al.*, 2001) or from axenic cultures under laboratory conditions (Staats *et al.*, 1999; Staats *et al.*, 2000; de Brouwer & Stal, 2002; de Brouwer *et al.*, 2002; Wolfstein & Stal, 2002). According to Wigglesworth-Cooksey & Cooksey (2005), when performing chemical extractions of EPS from field samples, one has to take two important factors into account, i.e. the method by which the samples are processed and the EPS extraction method, making the interpretation of the results more difficult. Thus *in-situ* localization and characterisation of EPS components may be a helpful tool. Initially, cytochemical methods and electron microscopy were used by Daniel *et al.* (1987) to study EPS localization of 17 marine diatom species. These techniques are useful to

detect the presence of polysaccharides and their modifications, but do not supply information about the composition of carbohydrate monomers. Wustman *et al.* (1997) combined localization of EPS using FITC-conjugated lectins and other fluorochromes and chemically analyzed the various sequentially extracted fractions of EPS from the sheath-forming diatom *Amphora* and two species of stalk-producing diatoms.

Lectins are proteins or glycoproteins of non-immune origin, which bind to carbohydrates specifically but reversibly, and agglutinate cells or precipitate glycoconjugates (Song *et al.*, 1999). When coupled to fluorochromes, the specific affinity of the lectins becomes a useful tool to detect the presence of specific sugar moieties in the EPS. Rhodes (1998) used lectins to differentiate between various toxic species of *Pseudo-Nitzschia* from New-Zealand on the basis of their differential production of surface sugars. He also stated that changes in surface sugars may depend on geographical origin and/or environmental conditions. In a recent report, fluorophore-conjugated lectins were used to differentiate between several extracellular polymers produced by marine biofilm diatoms like *Navicula* and *Amphora*, and were helpful in studying cell-cell interactions (Wigglesworth-Cooksey & Cooksey, 2005)

DAPI (4',6-diamidino-2-phenylindole) is well known for its DNA binding properties. It also binds to polysaccharides by an unknown mechanism. Negatively charged polyelectrolytes and dextran sulphate (a sulphated glycan) form a fluorescing complex with DAPI, yielding a blue emission. DAPI is also useful to detect polyphosphate depositions in the cells (Wustman *et al.*, 1997; Kawaharasaki *et al.*, 1999).

DTAF (5-(4,6-dichlorotriazinyl)aminofluorescein) reacts directly with polysaccharides and peptides at room temperature at pH above 9, so that it is useful for labelling natural organic compounds without disturbing their natural shape (Schumann & Rentsch, 1998).

In the present work, we used FITC (fluorescein isothiocyanate) labelled lectins, DAPI and DTAF to study the localization of EPS, and to identify sugar moieties in the EPS components from freshwater diatoms isolated from epilithic biofilms from littoral zone of Lake Constance.

## Material and Methods

### Isolation and culture conditions

Eighteen diatom isolates comprising 17 species (Table 2) were isolated from biofilms growing on stones at depths of 20-40 cm in the littoral zone (near the Limnology department, (47°41' N, 9°11' E) University of Konstanz) of Lake Constance (South Germany). Unialgal, non-axenic diatom cultures were established by repeated screening and were maintained on Diatom Medium (DM) (Watanabe, 2005) at 16°C at a 16:8 light/dark cycle using cool-white fluorescent tubes (50  $\mu\text{mol photons/m}^2 \text{ s}$ ).

### Staining procedure

Six different types of lectins conjugated to FITC, as well as DAPI and DTAF (all from Sigma-Aldrich, Munich) were used (Table 1) to study their binding to EPS secreted by diatoms.

Table 1: Specification of lectins, DAPI and DTAF used in this study

No.	Name	Affinity for	Origin	Control sugar
1	Con A	$\alpha$ -D-Man, $\alpha$ -D-Glc	<i>Canavalia ensiformis</i>	Glucose/Mannose
2	HAA	$\alpha$ -GalNAc	<i>Helix aspersa</i>	GalNAc
3	PSA	$\alpha$ -D-Man	<i>Pisum sativum</i>	Mannose
4	LEA	$\beta$ -GlcNAc	<i>Lycopersicon esculentum</i>	GlcNAc
5	WGA	GlcNAc	<i>Triticum vulgare</i>	GlcNAc
6	UEA I	L-Fuc	<i>Ulex europaeus</i>	Fucose

No.	Name	Affinity	Chemical name
7	DAPI	DNA, Polymeric substances	4',6-diamidino-2-phenylindole
8	DTAF	Carbohydrates, Proteins	5-(4,6-Dichloro-s-triazin-2-ylamino)fluorescein

Those diatoms that attach firmly to the substratum were grown up to the stationary phase in special chamber culture slides (BD Biosciences, Belgium) for two

days. The slides were washed twice with DM to remove unattached cells. For those diatom species that do not attach firmly to the slide (e.g. *Melosira* isolate number D-30 and *Fragilaria* isolate number D-78), 1 ml of each stationary phase culture was centrifuged at 100g for 5 min and the pellets were used for staining. The same staining procedure was used for staining with lectins, DAPI and DTAF, but the concentrations and times of incubation were modified. Lectins were used at a concentration of 0.1 mg ml<sup>-1</sup> in DM and incubated for 2 h, whereas DAPI (0.1 mg ml<sup>-1</sup>) and DTAF (0.1 mg ml<sup>-1</sup>, pH 9.5) were incubated for 30 min and 12 h, respectively. Slides with biofilms were washed in DM (pH 7.2), the staining solution was added and the slides were incubated at room temperature in the dark. Cell pellets were first washed with DM, and then staining was performed in microcentrifuge tubes. Afterwards the cells were washed twice with 1 ml DM to remove unbound dye, and left for another 5 min in DM. After this treatment, the chambers were removed and the slides were mounted in DM. Cell pellets stained in centrifuge tubes were diluted with 200 µl of DM and mounted on slides. Each experiment was repeated three times.

To ensure the specificity of the different lectins, we performed several controls. First, we pre-incubated the lectins at a final concentration of 0.1 mg ml<sup>-1</sup> for 1 h at room temperature with various concentrations of the corresponding carbohydrates as shown in Table 1 at concentrations of 0.1, 1.0, 5.0 and 10.0 mg ml<sup>-1</sup> before labelling. In addition, we used carbohydrates that are not specifically recognized by the respective lectins. In competition experiments, we were able to chase already EPS-bound lectins by the addition of the corresponding carbohydrates at final concentrations of 5.0 to 10.0 mg ml<sup>-1</sup>.

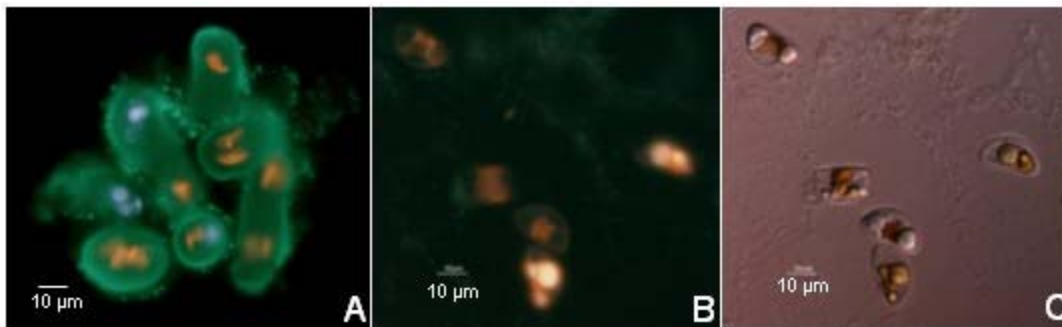
### **Microscopy**

Stained diatoms were observed by fluorescence microscopy with an Olympus BX51 fluorescence microscope equipped with a Nikon DMX-1200 camera using the filter sets HQ480/20 for DTAF and FITC-labelled lectins, UMF-2 for DAPI and U-MWSG2 for chlorophyll (Olympus, Hamburg) or a Confocal Laser Microscope (CFLM) (Model no LSM 510, Carl Zeiss, Germany with LSM 510 software) with a C-Apochromat 40x/1, 2 W objective.

## Results and Discussion

The formation of EPS structures by diatoms is an important feature of biofilm formation. They are mainly useful for attachment of the diatoms to the surface and to each other, but they may also serve as a nutrient source for heterotrophic bacteria (Stal & Défarge, 2005). In this study, we characterized EPS structures from diatoms by specific binding of fluorophores like FITC-conjugated lectins, DAPI and DTAF. We chose several diatom species that we found to be dominant in biofilms of the littoral zone of Lake Constance.

No lectin labelling was observed when the all lectins were preincubated with corresponding monosaccharides. We also observed that addition of specific sugars, result in removal of bound lectin within 1 h incubation when concentration (5-10 mg ml<sup>-1</sup>) of respective sugar was used. (Fig. 1)



**Fig. A-C.** Example for control experiments to analyze the specificity of lectin labeling. (A) Cells of *Cymbella caespitosa* (D-52) were incubated with the respective lectin-FITC conjugate PSA. Red fluorescence is due to chlorophyll autofluorescence, while the lectin-conjugate shows green fluorescence. (B) The PSA lectin was preincubated with the respective sugar (Mannose) before incubating with EPS, showing no subsequent staining of EPS structures. (C) Light microscopy image of (B)

The lectins demonstrated the presence of different sugar moieties in EPS structures like pads, capsules, and tubes that were secreted by the diatom isolates. The formation of EPS structures by diatoms is an important feature of biofilm formation. It is mainly useful for attachment of the diatoms to the surface and to each other, but it may also serve as a nutrient source for heterotrophic bacteria (Stal & Défarge, 2005).

The application of DAPI, DTAF and lectin staining techniques to study the localization and composition of diatom EPS has been proven to be useful in a several ways. Firstly, it yields basic information regarding the composition of sugar monomers of the respective diatom EPS; Secondly, it allows the visualization of

changes within the structures. Two main approaches have been used by several authors to elucidate structure and composition of diatom EPS, namely, *in situ* localization and chemical analysis. Localization or *in situ* studies focused on cytochemical staining methods (Daniel *et al.*, 1987), lectins (Wustman *et al.*, 1997; Rhodes, 1998; Wingglesworth-Cooksey & Cooksey, 2005) and microscopy (Higgins *et al.*, 2000; Wang *et al.*, 2000; Higgins *et al.*, 2002). Chemical analysis involved sequential isolation of EPS by treating with hot water, hot carbonate, EDTA and other solutions. Each fraction was then analyzed separately to elucidate the composition of EPS, either from sediment (Underwood & Smith, 1998; de Brouwer & Stal, 2001) or from laboratory-grown axenic cultures (Wustman *et al.*, 1997; de Brouwer & Stal, 2002).

In all species studied in this work, DAPI stained nuclei and frustules, however, labelling of EPS was different in various species (Table 2). DTAF mainly stained the frustules and in very few cases it was useful to stain EPS. FITC-conjugated lectins were the most useful fluorochromes for differentiating the structure of EPS.

**Table 2.** Staining of freshwater diatom species from Lake Constance by six lectins, DAPI and DTAF (see Table 1 for specificity of lectins).

Species (strain)	Type of EPS	Con A	PSA	HAA	WGA	LEA	UEA	DAPI	DTAF
<b>Adhering film or capsule forming diatoms</b>									
<i>Caloneis alpestris</i> (D-62)	CAE	+	+	+	+	+	+	+	+
	EPS	+	+	+	+	+	+	+	+
<i>Staurosira construens</i> (D-20)	CAE	-	-	-	-	-	-	+	+
	Capsule	-	-	-	-	-	-	+	-
<i>Achnanthes minutissima</i> (D-98)	CAE	+	-	-	-	-	-	+	+
	Capsule	-	-	-	-	-	-	+	-
<i>A. minutissima</i> (D-103)	CAE	+	-	+	-	-	-	+	+
	Capsule	-	-	+	-	-	-	+	-

<b>Tube forming diatoms</b>									
<i>Cymbella caespitosa</i> (D-52)	CAE	+	+	-	-	+	-	+	+
	Tube / Capsule	+	+	-	-	-	-	-	-
<i>Cymbella microcephala</i> (D-23)	CAE	+	-	-	-	-	-	+	+
	Tube / Capsule	-	-	-	-	-	-	+	-
<b>Pad forming diatoms</b>									
<i>Synedra angustissima</i> (D-16)	CAE	+	+	+	+	+	+	+	+
	Pads	+	-	+	+	+	+	+/-	-
<i>Synedra ulna</i> (D-33)	CAE	+	-	+	+	-	-	+	+
	Pads	+	-	+	+	+	-	-	-
<i>Asterionella ralfsii</i> (D-44)	CAE	+	+	+	+	+	+	+	+
	Pads	+	+	+	-	+	+	-	+
<i>Diatoma tenuis</i> (D-45)	CAE	+	+	+	+	-	-	+	+
	Pads	+/-	+	+	-	-	-	-	+
<i>Fragilaria capucina</i> (D-78)	CAE	+/-	+	+/-	+	+	-	+	+
	Pads	+	+	-	+	+	-	+	-
<i>Melosira varians</i> (D-30)	CAE	+	-	-	-	-	-	+	+
	Pads	+	+	+/-	-	-	-	+	-
<i>Fragilaria vaucheriae</i> (D-113)	CAE	+	+	-	-	-	-	+	+
	Pads	+	-	+	+	+	-		+
<i>Fragilaria</i> sp. (D-137)	CAE	+	+	-	+	+	-	+	+
	Pads	+	-	-	-	-	-	-	-
<b>Stalk forming diatoms</b>									
<i>Gomphonema truncatum</i> (D-124)	CAE	+	-	-	+	-	-	+	+
	Stalk-collar	-	-	-	-	-	-	+	-/+
	Stalk-footpads	-	-	-	+	-	-	+	+

	Stalk- middle part	-	-	-	-	-	-	+	-
<i>Cymbella</i> <i>cistula</i> (D-150)	CAE	+	-	-	-	-	-	+	+
	Stalk-collar	+	-	-	-	-	-	+	-
	Stalk- footpads	+	-	-	-	-	-	+	-
	Stalk- middle part	+	-	-	-	-	-	+	-
<i>Gomphonema</i> <i>olivaceum</i> (D- 140)	CAE	+	-	-	-	-	-	+	+
	Stalk-collar	+	-	-	-	-	-	+	-
	Stalk- footpads	+	-	-	-	-	-	+	-
	Stalk- middle part	+	-	-	-	-	-	+	-
<b>Other</b>									
<i>Amphora</i> <i>ovalis</i> (D-04)	CAE	-	-	+	+	+	+	+	+
	EPS	-	-	-	-	+	-	-	-

+ Staining observed, - No staining observed, +/- variable

### Capsules

Adhering films / capsules consist of amorphous EPS secreted by prostrately attached diatoms. Capsule secretion is fundamentally different from stalk and pad formation (Hoagland *et al.*, 1993) and it might influence also the attachment capabilities of the cells. Here, we report on a well-developed capsule formation in two isolates of *Achnanthes* (D-98 & D-103) and *Staurosira* (D-20). In earlier reports, the presence of a thin capsule was demonstrated in *Achnanthes lanceolata* (Rosowski *et al.*, 1986). Wustman *et al.* (1997) presented the localization of sugar moieties and biochemical analysis of stalks from *Achnanthes longipes*. To our knowledge, no reports are available on capsule formation by *Staurosira*. The capsulated cells of both *Achnanthes minutissima* Kützing (isolates D-98 and D-103) and *Staurosira construens* Ehrenb. (D-20) were loosely attached to the substratum surface. Therefore, capsule secretion might not play a role in firm attachment to the surface. No lectin binding to capsules and CAE of *S. construens* was observed, whereas staining of capsules by HAA and of frustules of *A. minutissima* (D-103) by Con A (Fig. 1A) and

HAA suggested the presence of  $\alpha$ -GalNAc in capsules and  $\alpha$ -GalNAc, Glc and /or Man in the CAE (Fig. 2A). Interestingly, we observed that DAPI stained capsules and CAE (D-20 Fig. 2B) of all three diatom isolates; it also stained polyphosphate particles, which appeared yellow (Kawaharasaki *et al.*, 1999).

We found that *Caloneis alpestris* (Grunow) Cleave (D-62) attached firmly to the substratum although no well-developed capsule formation was observed. All used fluorochromes showed fluorescence to the EPS and to the CAE of *C. alpestris* (D-62), suggesting a rather complex nature of the EPS. *C. alpestris* also produced long thread-like structures between distant cells, as well as gliding trails (Fig. 2C).

### *Tubes*

In the case of tube-forming diatoms, microscopic colonies may contain thousands of cells within a thick, tubular layer of mucilage. In such a tube cells are arranged in a row and are capable of moving within the tube (Cox, 1981). In *Cymbella cf. caespitosa* (Kützing) Brun (D-52), capsules and tubes were stained by Con A and PSA (Fig. 2D-F) suggesting the presence of Man and Glc moieties. In contrast, capsules and tubes of *C. cf. microcephala* Grunow (D-23) were not stained by any of the used lectins. The capsule and tubes of *C. caespitosa* (D-52) were not stained by DAPI, however, capsules and tubes of *C. microcephala* (D-23) were stained by DAPI only. In *C. microcephala* (D-23), capsules stained by DAPI showed higher fluorescence intensity than tubes. Interestingly, labelled tubes revealed ring-like structures connected to each other (Fig. 2G and H). Tubes produced by *C. caespitosa* (D-52) were of the same thickness throughout and contain living cells. In our study, *C. caespitosa* (D-52) indicated the presence of Man and Glc by binding to the lectins Con A and PSA. Chemical analysis of the tube-forming diatom *Berkleya* revealed the presence of sulfated 3-linked mannuronosyl, 4-linked 3, 6-anhydroglucosyl residues and proteins (Smestad-Paulsen *et al.*, 1978). However, there are no other reports available on the biochemical nature of tubes from freshwater diatoms.

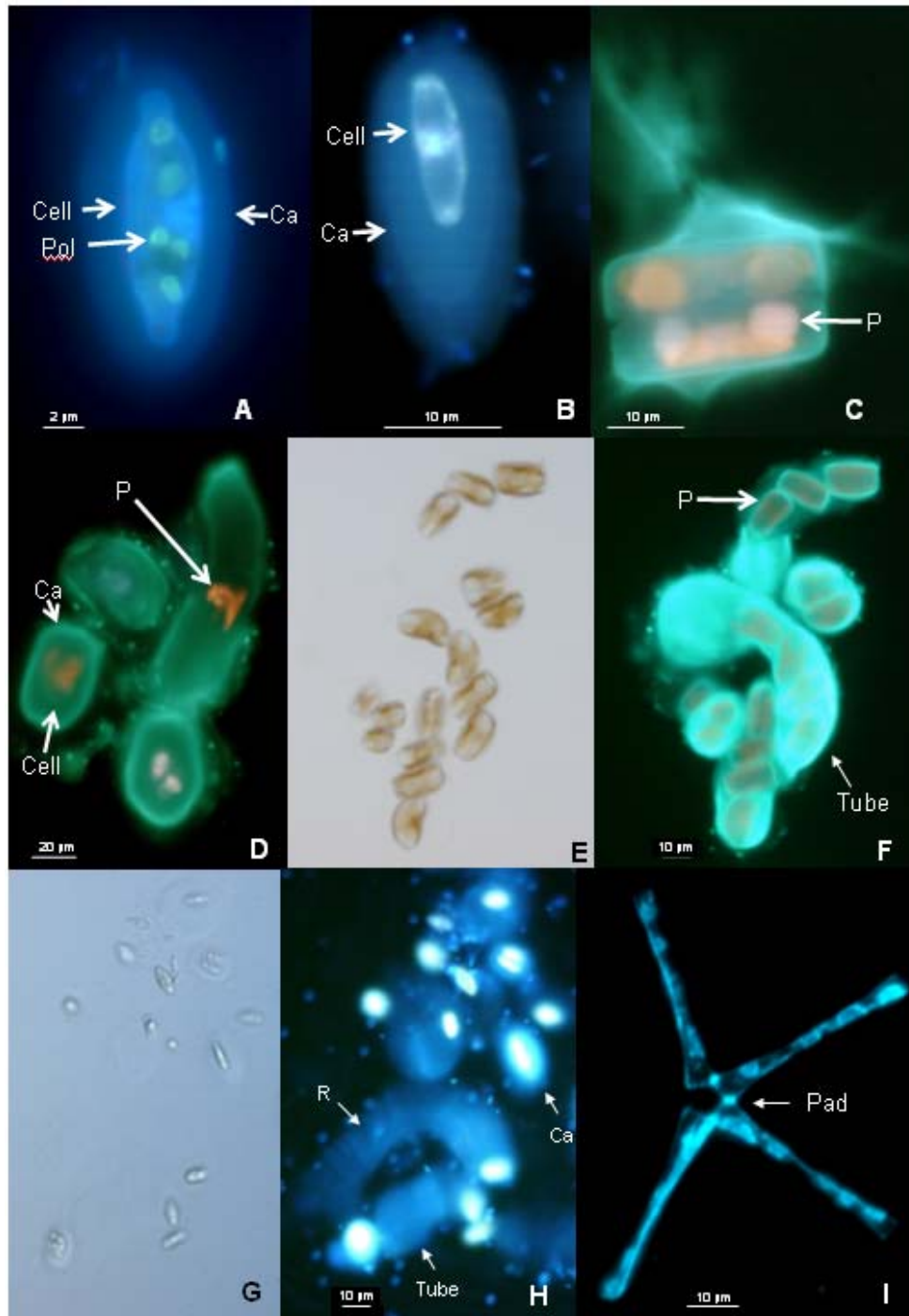


Fig. 2 Staining of various diatoms by FITC-conjugated lectins and DAPI (A) DAPI stained *Staurosira* (D-20) cell showing a capsule; (B) Capsule of *Achnanthes* (D-98) stained by DAPI; (C) *Caloneis alpestris* (D-62) showing EPS stained by HAA, plastids appeared due to chlorophyll autofluorescence; (D) Capsules and tube with two celled of *Cymbella caespitosa* (D-52) stained by PSA; (E)(F) tube of *C. caespitosa* : (E) Light-microscopical image of several cells with the tube like structure and (F) staining of same tube with PSA; (G, H) Capsule and tube like structures of *C. microcephala* (D-23) : (G) Light-microscopical image (H) EPS stained by DAPI; (I) Star shaped colony of *Asterionella ralfsii* (D-44) labelled with HAA, Arrow pointing to pad. The scale for E and G is identical to F and H, respectively. Abbreviations: Ca, capsule; P, Plastid; Pol, putative polyphosphates; R, ring-like structures.

During, tube formation in *C. caespitosa* (D-52) and *C. microcephala* (D-23) we identified different stages giving the impression that tube formation is an interesting and highly ordered sequential process. We had the impression that initially after the capsules are formed, they first elongate before the cells start to divide. Separation of the daughter cells then leads to an additional elongation of the capsule, resulting in a tube-like structure. This tube formation continues and multi-celled tubes are formed (Fig. 2E and F).

### *Pads*

Many diatoms in biofilms produce small globular structures of EPS which are useful for cell-to-cell attachments or for attachment to the substratum. Species producing cell-to-cell attachments form long chains of cells or small colonies. To maintain integrity of the colony, the cells may secrete pads and intercellular layers.

Apical pads are frequently similar to short stalks with respect to their site of secretion at the apical pore field (APF) (Hoagland *et al.*, 1993). *Asterionella ralfsii* W. Smith mainly is a planktonic diatom, thus occurrence of it in biofilms on stones might be due to the effect of water turbulence. It is mainly observed in the water column and its colony shape may assist to prevent it sinking (Hoagland *et al.*, 1993). Different *Asterionella* species produce two pads on each wall face and eventually star-shaped colonies are formed (Hoagland *et al.*, 1993). Pads and CAE of *A. ralfsii* (D-44) were labelled by all lectins used (Fig. 2I) and by DTAF, suggesting a heterogeneity of the EPS and a similarity between EPS around the frustules and within the pads. Hecky *et al.* (1973) showed the presence of Rha, Man and Fuc in frustules of *A. ralfsii*. In our study, lectin staining indicated the presence of Man (PSA and Con A) and Fuc (UEA I). Presence of a fibrous EPS coat around cells of *A. formosa* was reported by Hoagland *et al.* (1993). In our study, such a coat was labelled by all lectins.

*Synedra* cells usually attach perpendicular to the substratum by secreting basal pads. Subsequent cell divisions lead to the formation of radiating fan-like colonies. Pads of freshwater *S. acus* were reported to contain polysaccharides and amino acids (Watt, 1969). Daniel *et al.*, (1987) demonstrated the highly sulfated nature of the *Synedra affinis* pads while *S. ulna* pads contained sulfated/carboxylated carbohydrates (White & Chamberlain, 1982). However, there are no reports available on the monosaccharide composition of pads of *Synedra*. In our study, lectin staining demonstrated presence of Glc, Man (Con A and PSA) (Fig. 3A, B),  $\alpha$ -GalNAc

(HAA) and  $\beta$ -GlcNAc (WGA) in the pads of both species. Fig. 3 B showed the impression of the frustule where a cell was attached previously and unattached cells can have pads at both ends of the cell (Fig 3A). Presence of Fuc was detected in *S. angustissima* by staining with lectin UEA I, which was absent in the CAE of *S. ulna* (Nitzsch) Ehrenberg. All lectins stained frustules of *S. angustissima* (Fig. 3 C) whereas Man and Fuc were apparently absent in *S. ulna*. In *Melosira varians* Agardh (D-30), the filamentous nature of the EPS is a characteristic feature, and was maintained or preserved due to presence of intercellular pads or layers. Cell walls of two species of *Melosira* were mechanically isolated by Hecky *et al.* (1973). The authors demonstrated differences in the monosaccharide composition between cell walls of two species with of Glc, Xyl, Man and Fuc in different concentrations. Freshwater species have relatively low amounts of Fuc when compared with estuarine species (Hecky *et al.*, 1973). In accordance, our isolate showed the presence of Glc and Man (Con A and PSA Fig. 3D) while no Fuc (UAE I) was detected. Additionally, the presence of sulfated polysaccharides in the intercellular pads of *Melosira* has been reported earlier (Daniel *et al.*, 1987).

*Diatoma tenuis* Agardh (D-45) produces characteristic zigzag colonies with two apical pads on the opposite valve faces. Pads showed the presence of Glc, Man (Con A, Fig. 3E) and GlcNAc. However, White & Chamberlain (1982) detected the presence of sulfated polysaccharides in *D. vulgaris*. Beyond these, there are no reports available on localization of EPS structure in *Diatoma*.

Different colony morphologies were observed in the three *Fragilaria* species; long chain formation mediated by the EPS layer was observed in *F. capucina* Desmazères (D-78), zigzag colonies in *F. vaucheriae* (Kützing) Patersen (D-113) and stellate colonies in *Fragilaria* sp (D-137). In pads of *F. capucina*, the presence of Man, Glc and  $\beta$ -GlcNAc (Table 2, Fig. 3F) was suggested by lectin binding. In *F. vaucheriae*, pads between young cells were stained by Con A, HAA, WGA and LEA whereas in *Fragilaria* sp. (D-137) pads stained by Con A suggested the presence of Glc and/or Man only. To our knowledge, no reports on the chemistry of *Fragilaria* EPS are available for comparison. Basal attachment and intercellular adhesives in a *Fragilaria* species from marine water contain acidic polysaccharides bearing both carboxyl and sulfated groups in various proportions (Daniel *et al.*, 1987).

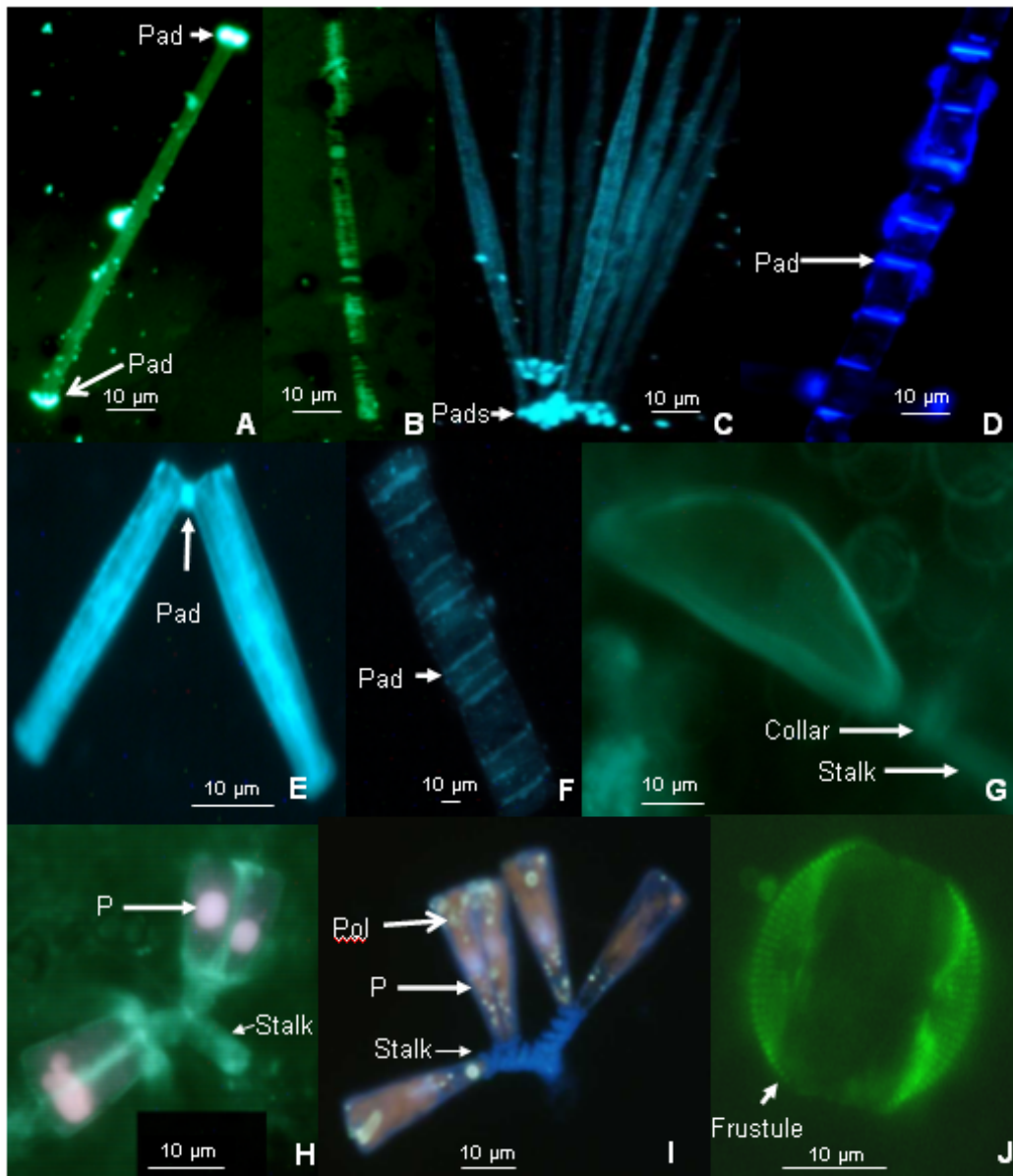


Fig. 3 Staining of various diatoms by FITC-conjugated lectins and DAPI. (A) HAA binding showing presence of pads at both ends of the cell of a non-attached cell of *Synedra ulna* (D-33); (B) HAA staining to the carbohydrate impression of a previously attached cell of *S. ulna* (D-33); (C) HAA stained colony of *Synedra angustissima* (D-16) showing the presence of basal pads; (D) Intercellular pads and EPS on the sides of cells of *Melosira variance* (D-30) stained by PSA (E) Pads of *Diatoma tenuis* (D-45) stained by PSA; (F) Intercellular pads of *Fragilaria capucina* stained with Con A; (G) Con A binding to all parts of *Cymbella cistula* (D-150) showing presence of small collar and stalk; (H) Con A staining to the stalk and intercellular EPS of the *Gomphonema olivaceum* (D-140); (I) Staining of stalk of *Gomphonema truncatum* (D-124) by DAPI; (J) Cell wall associated EPS of *Amphora ovalis* (D-04) stained by LEA. Abbreviations: P, Plastid; Pol, putative polyphosphates.

### Stalks

Stalks are the elongated mucilaginous unidirectionally deposited, multilayered, physical structures attaching diatom cells to a substratum (Hoagland *et al.*, 1993). Here we studied two genera and three species of the stalk forming diatoms *Cymbella* sp., *Gomphonema truncatum* Ehrenberg and *G. olivaceum* Hustedt. Long slender and unbranched stalks were produced by *C. cistula* and *G. olivaceum*, whereas they were branched in *G. truncatum*. Many *Gomphonema* and *Cymbella* species have apical pore fields (APF), from which stalks are produced. Species lacking APF do not produce stalks. Dawson (1973), suggested that presence of APF is a main requirement for stalk production.

Biochemical analysis of stalks of freshwater *Cymbella cistula*, *C. mexicana* and *Achnanthes longipes* by (Wustman *et al.*, 1997) demonstrated differences in the respective polysaccharide structures. In our study, in *C. cistula* (Ehrenberg) Kirchner (D-150) all parts of the stalk and the frustules were labelled by Con A only (Fig. 3G). However, according to Wustman *et al.* (1997), in *C. cistula* only frustules were stained by Con A and the stalk and frustules labelled by APA (specific to D-Gal) and UEA was reported to bind mucilage associated with the apical pore field. Xyl and Gal were reported in mechanically isolated stalks of *G. olivaceum* (Huntsman & Sloneker, 1971). Our study confirmed the presence of Man and / or Glc (Con A) moieties in *G. olivaceum* (D-140) (Fig. 3H), while a second isolate (D-124) showed the presence of GlcNAc (WGA) in frustules and footpads. Stalks of both diatoms also showed staining with DAPI (D-124 Fig. 3I)

### Other EPS forms

In *Amphora coffeaeformis*, a well-studied marine capsule-forming species, Con A was reported to stain EPS between cells and the organic sheath, and DAPI to label capsules (Wustman *et al.*, 1997). In our study, no EPS structures were stained by lectins in *A. ovalis* Kützing, but staining of frustules was observed by a number of lectins (Table 2, Fig. 3J). Cells were firmly attached to the glass surface and in contrast to *A. coffeaeforma*, no Con A and DAPI binding were observed.

In most of the studied diatoms, lectin-binding was useful to localize the EPS structures, indicating the presence of specific sugar moieties in the polysaccharides. Furthermore lectins were useful for comparing similar EPS structures between different diatom species. Although it is not possible to differentiate individual

carbohydrates by staining with DAPI and DTAF, these fluorochromes are especially useful to highlight the structure of the EPS in those cases where lectins did not bind.

We repeated all experiments thrice and found the same labelling patterns with the individual diatom strains, indicating that (i) labelling with the lectin is specific for certain monosaccharides and (ii) there is no considerable change in the composition of the EPS over time in a given species. However, it cannot be excluded that under certain environmental conditions the individual monosaccharide content of the EPS structures may change.

In conclusion, our study gives an insight into localization and composition of EPS structures formed by diatoms isolated from epilithic biofilms from a freshwater system. Although it does not provide the exact chemical composition of the complex EPS, it gives us an idea of the sugar monomers present and also shows the structural details of the EPS which are difficult to visualize by light microscopy.

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## Chapter 6

### Changes in the concentration of extracellular polymeric substances of freshwater diatom species from Lake Constance (Germany)

Rahul A Bahulikar and Peter Kroth

1. Faculty of Biology, University of Konstanz, University str. 10, Konstanz Germany

Key words: benthic diatoms, EPS, chlorophyll *a*, monosaccharide composition

Abbreviations: BE, bound EPS; CC, cellular carbohydrates; EPS, extracellular polymeric substances; RC, residual carbohydrate; SE, soluble EPS;

**Abstract**

This study deals with the diatom growth and dynamics of extracellular polysaccharides (EPS) secreted by twelve different diatom isolates. Most of these isolates were co-dominant diatom species in epilithic biofilms of Lake Constance including three species of *Cymbella* (7 isolates), *Achnanthes minutissima* (3 isolates) and *Pseudostauroopsis* (2 isolates). Growth of these isolates was measured by analysis of chlorophyll *a*. EPS was fractionated into soluble and bound fractions. Intracellular storage carbohydrates and residual carbohydrates were also analyzed. Growth rates and the maximum concentrations of EPS were different in various isolates of *C. microcephala*. In some *Cymbella* species and in *Achnanthes minutissima*, comparatively lower growth rates as well as lower amounts of the soluble EPS (SE) were observed compared to *C. microcephala*. Two isolates of *Pseudostauroopsis* showed variable growth rates and also a lower SE content. Isolates of *C. microcephala* showed the highest SE content compared to all other species, where the residual carbohydrate content was higher than in bound and cellular carbohydrate fractions. Monosaccharide profiles of the SE fractions were analysed. In all three species of *Cymbella* a high amount of galactose while in the three isolates of *Achnanthes* more mannose/xylose was observed, whereas in *Pseudostauroopsis* a heterogeneous composition was found. The monosaccharide composition of pore water from the biofilms was rather heterogeneous. In principal component analysis, genera-specific monosaccharide signatures were observed and monosaccharide profiles of pore water were grouped with the profiles of *A. minutissima*.

## Introduction

Diatoms are the most diverse group of eukaryotic organisms and may contain more than 100,000 species (Mann, 1999). They are found in both freshwater and marine environments as well as on wet surfaces (Medlin, 2002; Punning et al., 2004; Underwood & Paterson, 1993). They are adapted to two different living conditions, either as free floating (planktonic) or attached (benthic) (Alekseeva et al., 2005). They are important primary producers and CO<sub>2</sub> fixed by diatoms in the oceans may count up to ~40% of total primary production (Mann, 1999). Fixed carbon is used as structural carbohydrates or stored in the form of chrysolaminaran or secreted outside the cells as extracellular polymeric substance (EPS) (Hoagland et al., 1993). Approximately 20-80% of the fixed carbon is secreted as polysaccharides as a metabolic overflow (Goto et al., 2001). In addition, proteins (Staats et al., 1999), glycoproteins (Chiovitti et al., 2003), uronic acids (Chiovitti et al., 2003; de Brouwer & Stal, 2002; Staats et al., 1999; Wustman et al., 1997) also contribute to a small fraction of EPS. EPS plays an important role in the life cycle of diatoms and may greatly influence the ecosystem. EPS secretion depends on oxygenic photosynthesis and usually a strong secretion occur mainly during the daytime (Staats et al., 2000). EPS can be released in the water or remain adhered to the cells. Based on these properties, the exudations are classified as soluble or bound EPS. Soluble EPS (SE) contains small molecular weight polysaccharides which are released into the surrounding water (de Brouwer & Stal, 2002; Hoagland et al., 1993). Bound EPS (BE) mainly contains high molecular weight polysaccharides, remains adhered to the diatom cells (de Brouwer & Stal, 2002; de Brouwer & Stal, 2004) and fulfils various functions. EPS gives protection to the silicious walls, protects cells from herbivores, holds pregametangial cells together and raises the cells from the surface of substrata to avoid competition for light and nutrients (Hoagland et al., 1993).

Ecologically, EPS mainly serves as food for other organisms like bacteria (Giroldo et al., 2003). It holds the sediment particles together which results in lower erosion (de Brouwer et al., 2005; Stal, 2003; Yallop et al., 2000). Biofouling effects are caused by a very thin layer of biofilm developed on the immersed surface of ships. It decreases the hydrodynamic nature of ships, which causes loss of performance and a high consumption of fuel (Chiovitti et al., 2003).

Diatoms are the dominant members of benthic biofilms (Ács et al., 2000; King et al., 2000; Underwood, 1994; Underwood & Paterson, 2003) and are also known as early colonizers of the surfaces (Ács et al., 2000; Barbiero, 2000). Our earlier reports demonstrated the community structure of diatoms at a small depth gradient and on a temporal scale (Chapters 2 and 3). The concentration of SE in the pore water was also measured in these studies. In both the reports, dominance of *Achnanthes minutissima* (For authorities please refer Table 1), *Cymbella microcephala*, *C. minuta* and *Pseudostauroopsis* was noted. Due to their numerical dominance in the biofilms, these species might contribute substantially to the EPS content in the pore water of the biofilms. Therefore, with the help of these isolated species, we tried to answer the following questions:

1. Which species produce more EPS under culture conditions?
2. Is there any difference in the EPS production in isolates of the same species under identical growth conditions like light intensity, temperature, nutrients etc.?
3. Is there any difference in the monosaccharide composition at species or generic level?
4. Is there a correlation between the monosaccharide composition of biofilm EPS and the composition of SE produced by diatom species under axenic condition.

## Material and Methods

### *Description of collection site*

Lake Constance is a mesotrophic, phosphorous limited and hard water prealpine lake (Schmieder et al., 2005). The lake is divided in to two parts, the lower lake and the upper lake with a total surface area is 476 km<sup>2</sup> and maximum depth of 252 m (Rosenstock & Simon, 1993).

### *Isolation of organisms*

Various diatoms (Table 1) were isolated from epilithic biofilms of the littoral zone of Lake Constance (Germany, 47°41'N, 9°11'E). The cultures were maintained in diatom medium (DM) (Watanabe, 2005) at 16°C for 16 h and 50 µE light intensity. The illumination was provided by cool-white fluorescent tubes. Isolated strains were streaked on DM plates with combination of three antibiotics (10 µg/ml kanamycin, 10 µg/ml amikacin and 10 µg/ml erythromycin) for the elimination of bacterial contamination. Clean colonies were picked under a microscope. Bacterial contamination was checked by epifluorescence microscopy after staining the diatom isolates with SYBR Green I (Ambrex Bioscience, Germany) and streaking them on nutrient agar.

### *Experimental design*

Each axenic diatom culture (Table 1) was maintained in 100 ml flasks containing 75 ml of DM medium. Three ml of starter culture of each isolate was inoculated in 21 separate 100 ml flasks containing 75 ml of fresh DM and were grown under conditions as described before. Sampling was done after 4, 8, 12, 16, 20, 24 and 28 days each in triplicate. On each sampling day, 1 ml culture was used for a chlorophyll *a* assay (described below) and 1 ml was used for measuring concentration of the SE. The remaining culture was centrifuged at 5000 rpm for 10 min and spent medium was concentrated to the 1/10<sup>th</sup> of original volume using a rotary evaporator and precipitated by 4 volumes of ethanol (final concentration 80% alcohol) followed by overnight incubation at -20 °C. It was then centrifuged and subsequently dried under a flow of nitrogen or air-dried. Cell pellets were used for the extraction of different carbohydrate fractions as mentioned in the following paragraphs.

Table 1 comprises isolate number and colony morphology of the diatom species used in this analysis

Isolate no.	Species	Colony type
A-003	<i>Achnanthes minutissima</i> Kützing	Single cell
B-006	<i>Achnanthes minutissima</i> Kützing	Single cell
I-117	<i>Achnanthes minutissima</i> Kützing	Single cell
B-004	<i>Cymbella microcephala</i> Grunow	Single cell
B-008	<i>Cymbella microcephala</i> Grunow	Single cell
I-026	<i>Cymbella microcephala</i> Grunow	Single cell
D-023	<i>Cymbella microcephala</i> Grunow	Single cell
I-147	<i>Cymbella minuta</i> Hilse	Single cell
I-051	<i>Cymbella minuta</i> Hilse	Single cell
I-034	<i>Cymbella vulgata</i> Krammer	Single cell
F-3	<i>Pseudostauroopsis</i> Williams & Round	Chain
Dx7	<i>Pseudostauroopsis</i> Williams & Round	Chain

#### *Chlorophyll a analysis*

Chlorophyll *a* content was used to monitor diatom growth. For determination, 1 ml of homogenized culture was centrifuged and chlorophyll *a* was isolated from cell pellet using methanol : acetone (90:10) after 15 min vortex or sonication, centrifuged at full speed and was measured spectrophotometrically according to Jeffrey & Humphrey (1975).

#### *Isolation and analysis of carbohydrate fractions*

Carbohydrates were measured using a phenol / H<sub>2</sub>SO<sub>4</sub> assay (Dubois et al., 1956). The procedure for the estimation of carbohydrates was used according to (de Brouwer & Stal, 2002) with some modifications. For extraction of the bound EPS (BE), cell pellets were resuspended in 2 ml of distilled water and incubated at 30 °C for 1 h under continuous stirring. Internal sugars were extracted by resuspension of the cell pellet in 0.05 M H<sub>2</sub>SO<sub>4</sub> (CC) and incubation for 2 h at room temperature with shaking this was followed by centrifugation. From the resulting supernatant 200 µl were used for the carbohydrate assay and the resulting cell pellets were suspended in

1 ml of distilled water (instead of 400  $\mu$ l) and from that 200  $\mu$ l was used for residual carbohydrate measurement (RC)

*Monosaccharide composition*

5 mg of precipitated and dried EPS was hydrolyzed with 2 M Tri-fluoro Acetic acid (30 min at 121°C). The composition of monosaccharides was analyzed by a HPLC equipped with carbopac PA10 column (Dionex Germany) and a pulse amperometric detector system (Jahnel et al., 1998). Chameleon software (Dionex, Germany) was used to analyze the individual runs.

*Carbohydrates from biofilms*

For EPS analysis from biofilms, 3-4 stones in close vicinity to each other were collected separately from 2 different places, from the littoral zone of Lake Constance. In the laboratory, biofilms were scraped from the surface of the stones and centrifuged at 5000 rpm for 10 min to separate biofilm from pore water. Pore water was precipitated, hydrolysed and analysed by HPLC as described before.

*Data analysis*

Principal component analysis (PCA) of the monosaccharide profiles of all isolates was done by MVSP software (Kovach, 2002).

## Results

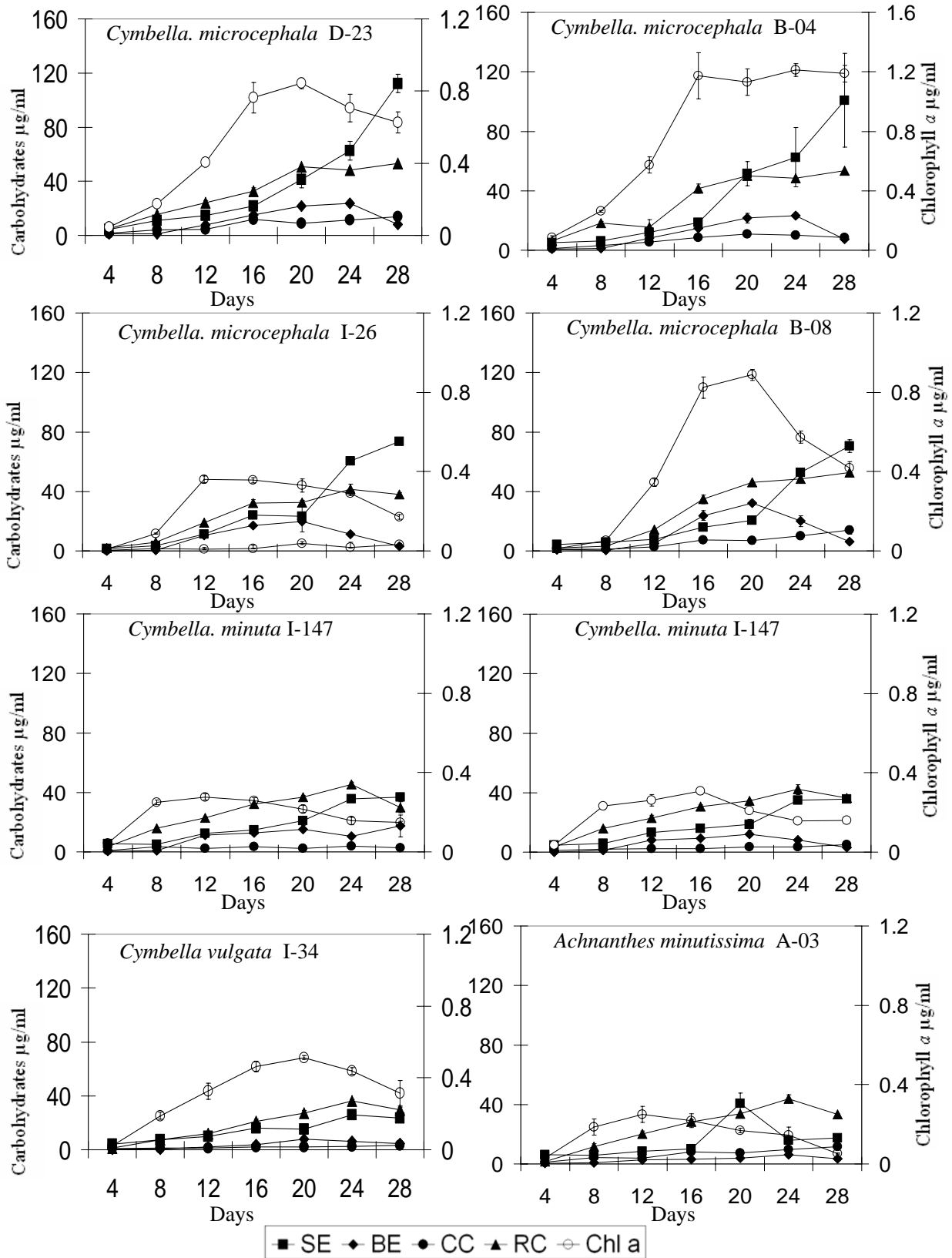
Seasonal fluctuations in the diatom community and changes in the SE and BE of the epilithic biofilms were studied earlier (Chapter 2 and 3). A very high correlation was observed between chlorophyll *a* content and both EPS fractions (soluble and bound). This suggests photosynthetic origin of EPS. As diatoms were dominant members in epilithic biofilms, we wanted to know which diatom species are responsible for high EPS concentrations. In the laboratory, dominant diatom members were isolated cultivated and used in this study.

Growth of various diatom isolates and the corresponding EPS production at various stages of growth was monitored. Here we studied, three species of *Cymbella*, namely *C. microcephala* (4 isolates), *C. minuta* (2 isolates) and *C. vulgata* (1 isolate), one species each of *Achnanthes minutissima* (3 isolates) and *Pseudostauroopsis* (2 isolates). Diatom growth was monitored measuring the chlorophyll *a* content. During growth of each isolate, we measured concentration of cellular carbohydrates (CC), SE, BE and residual carbohydrates (RC) from each time point.

Four isolates of *C. microcephala* showed variable growth rates under identical conditions such as light intensity, temperature, photo period and nutrients. In all the isolates, SE production was observed from the 8<sup>th</sup> day after inoculation. The concentration of SE increased gradually. A sharp increase in the SE concentration was observed from the 20<sup>th</sup> day and reached to a maximum of 73  $\mu\text{gml}^{-1}$  (B-08) – 112  $\mu\text{gml}^{-1}$  (D-23) (Fig. 1). Similar to variable growth rates, all the isolates showed differences in the maximum concentrations of the SE produced. BE concentrations were lower than that of SE and interestingly, an increase in the SE concentration were corresponding to decreased concentration of BE. Cellular carbohydrate (CC) reached to maximum concentration of 14.1  $\mu\text{gml}^{-1}$  (D-23, Fig. 1). The residual carbohydrate (RC) content was considerably higher than of BE and CC fractions during the experiment and it reached a maximum of 53.24  $\mu\text{gml}^{-1}$  (D-23). Monosaccharide composition of SE showed the presence of high amount of galactose (37-42%) in all isolates as a main sugar (Fig. 2).

Both the isolates of *C. minuta* showed lower growth rates than *C. microcephala*. However, no differences were observed in the growth rates as well as in the EPS production profiles of both isolates of *C. minuta*. SE content reached a maximum of 35- 36  $\mu\text{gml}^{-1}$  in both cases (Fig. 1). Very low BE and CC contents were

observed. The RC content was higher than in all the other fractions 29-36  $\mu\text{gml}^{-1}$  (Fig. 1). Monosaccharide composition of SE showed the presence of a high amount of galactose (52-53%) as a dominant sugar, which was slightly higher than



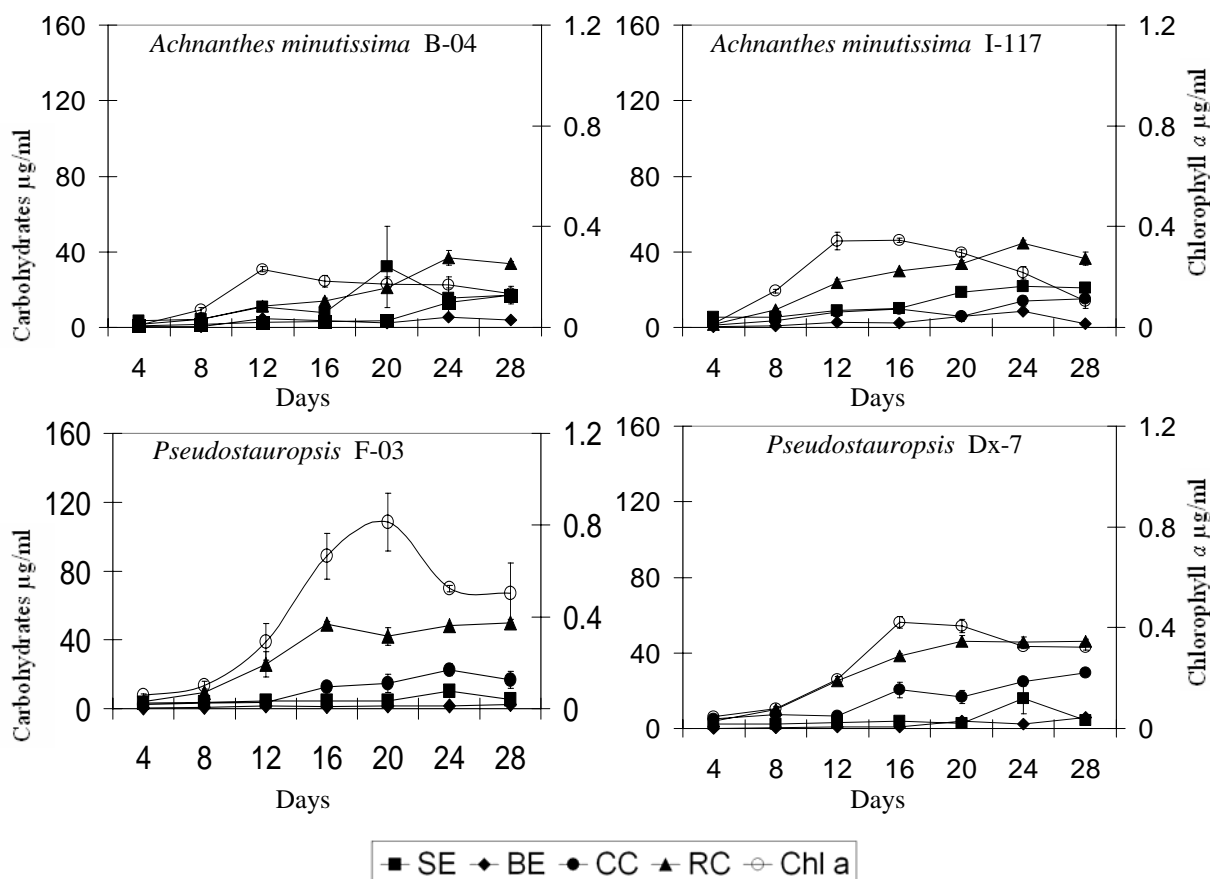


Fig. 1 Carbohydrate analysis of various diatom isolates. Each graph is showing species name and respective isolate number. On the primary Y-axis, concentrations of Soluble (SE) and bound EPS (BE), cellular carbohydrates (CC) and residual carbohydrates (RC) ranged from 0-160  $\mu\text{gml}^{-1}$ . The cell growth is in terms of chlorophyll *a* content ranged from 0-1.2  $\mu\text{gml}^{-1}$  on secondary Y-axis (In case of isolates B-04 it is 1.6  $\mu\text{gml}^{-1}$ ) were sampled at each time point (X- axis in days) of all studied isolates data points indicate mean value and error bars represents standard error (n = 3)

*C. microcephala* (Fig.2). The RC content was higher than in all the other fractions 29-36  $\mu\text{gml}^{-1}$  (Fig. 1). Monosaccharide composition of SE showed the presence of a high amount of galactose (52-53%) as a dominant sugar, which was slightly higher than *C. microcephala* (Fig.2).

In the single isolate of *C. vulgata*, the growth rate was slightly higher than for *C. minuta*, whereas carbohydrate concentrations in all fractions and monosaccharide patterns of SE fractions showed similar pattern as for *C. minuta* (Fig. 1). The only difference observed was the presence of arabinose instead of rhamnose in *C. minuta* (Fig. 2).

Growth rate and rate of EPS production in the 3 isolates of *A. minutissima* studied were similar to *C. minuta*. In monosaccharide analysis mannose/xylose and galactose were present in nearly equal concentrations.

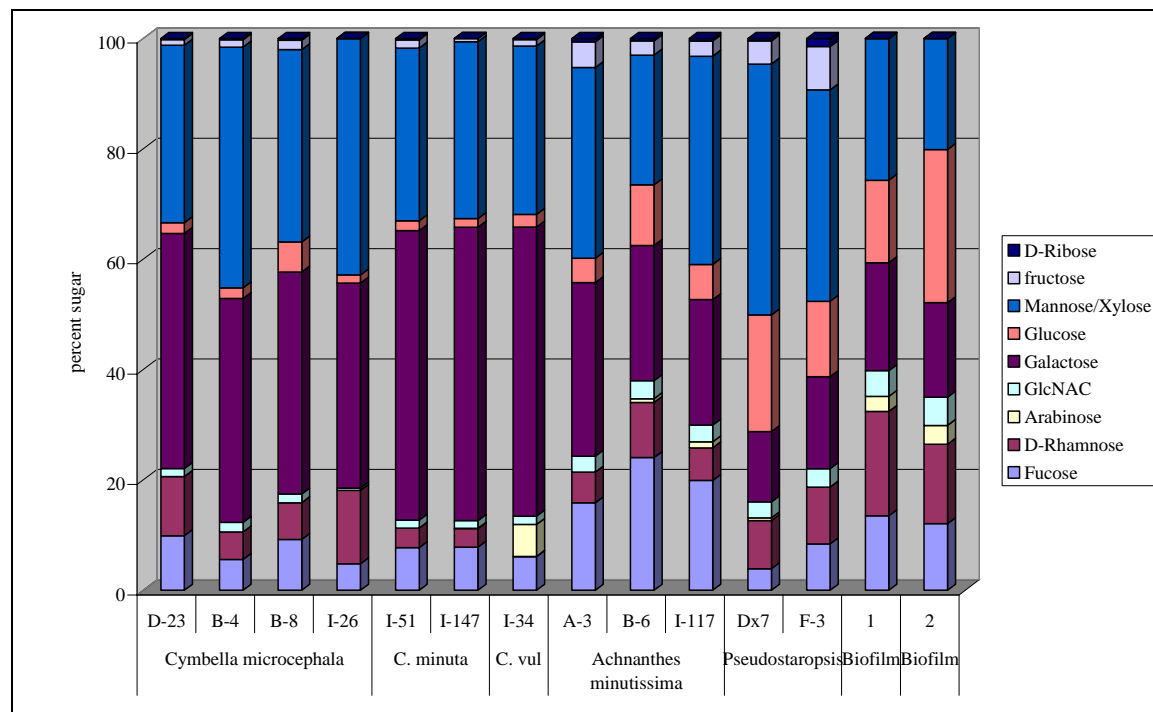


Fig 2 Monosaccharide compositions of the soluble EPS of all isolates compared with monosaccharide composition of pore water from biofilms details of isolate number are in Table 1. *C. vul*= *Cymbella vulgata*

As observed in *C. microcephala*, variable growth rate was observed in the two isolates of *Pseudostauropsis*, but in general showed a low EPS content in SE, BE and CC fractions. RC content was more and the values was 46-50  $\mu\text{gml}^{-1}$ ) (Fig. 1). In *Pseudostauropsis* mannose/xylose were present as dominant sugars (Fig. 2).

In general, only *C. microcephala* showed a higher growth rate and a very high SE production compared to the other species. Species specific monosaccharide composition was observed, whereas pore water analyzed from the biofilm showed a heterogeneous nature.

PCA of the monosaccharide profiles of the SE fraction from all isolates showed four groups (Fig. 3), Group I comprise of *Pseudostauropsis*, Group II contains both the biofilms and one isolate from *A. minutissima* (B-06). The third group showed all isolates from *C. microcephala* whereas, Group IV showed *C. minuta* and *C. vulgata* isolates. The remaining two isolates of *A. minutissima* can be

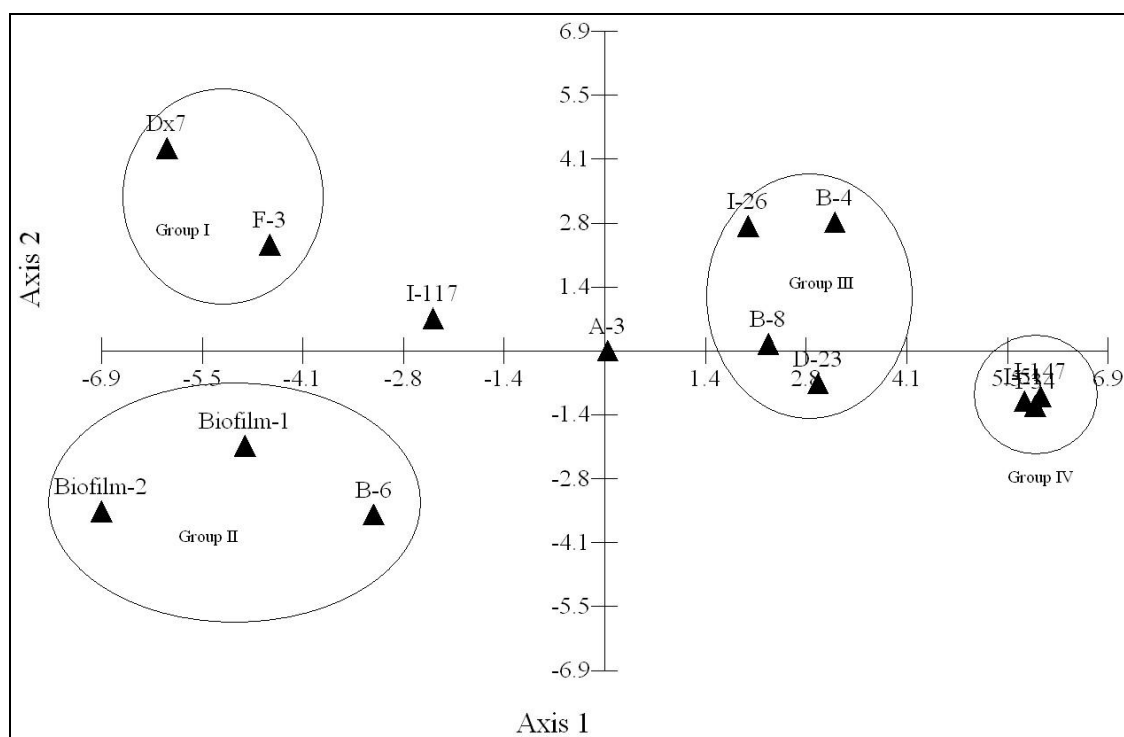


Fig.3 Principal component analysis showing species specific grouping from monosaccharide profiles of the soluble EPS fractions of the various diatom isolates details of isolate number are in Table 1

considered as related to either of the groups. Therefore, distinct separation was observed in the monosaccharide profiles of SE of all isolates and showed species-specific patterns.

## Discussion

Benthic biofilms from various water bodies such as intertidal regions (Azovsky et al., 2000; Mitbavkar & Anil, 2002), lakes (Ács, 1998; Ács & Buczkó, 1994) and streams (Soininen, 2004) are mostly dominated by diatoms. They are known to fix carbon photosynthetically and store it as chrysolaminaran and/or secrete it outside the cell as a metabolic overflow (Stal, 2003). Such exudates are either just released in the water which is referred as soluble EPS (SE) or used for various activities such as cell migration or permanent adhesion (Hoagland et al., 1993). SE can serve as source of energy for bacteria (Girollo et al., 2003) and also can be utilized by diatoms themselves in the dark (de Brouwer & Stal, 2002). Here we studied the dynamics of cellular, soluble and bound polysaccharides of 12 diatom isolates during a growth period of 28 days. Species used in this investigation were co-dominant diatom members in littoral epilithic biofilms (Chapters 2 and 3). We have also reported the presence of variable amounts of the SE in the pore water of epilithic biofilms at small depth gradients and biofilms collected at different seasons. Dominant diatoms might have an effect on the nature or the structure of biofilm as well as they also might contribute to the pore water EPS. Therefore, we isolated and cultivated specific diatom species for further investigations.

### *EPS production*

The EPS fractionation scheme of (de Brouwer & Stal, 2002) which included fractionation of carbohydrates into SE, BE, CC and RC was found to be useful to monitor the changes of concentrations during the growth period. Among all the studied diatoms, only isolates of *C. microcephala* produced high levels of the SE (73-112  $\mu\text{gml}^{-1}$ ) compared to produce by others. These concentrations were higher than reported for the marine diatoms *Cyloedrotheca closterium* and *Nitzschia* sp (de Brouwer & Stal, 2002) and *C. closterium* and *Nitzschia* sp. (Staats et al., 1999). The SE concentrations of other species from this report were in accordance with the above-mentioned two reports. We also studied growth and EPS production dynamics of unialgal non-axenic isolates such as *Diatoma tenuis*, *Gomphonema truncatum*, *Navicula cinta*, *Melosira*, *Nitzschia* and two species of *Synedra* but none of the showed such high amounts of SE content as in *C. microcephala* (data not shown).

Bound EPS (BE) normally remains attached to the cell wall or is also found as stalks, tubes and capsules like structures (Hoagland et al., 1993). In many studies,

loosely attached BE was extracted using warm water (30 °C) treatment (de Brouwer & Stal, 2002; Staats et al., 1999). BE was also found to be the highest in *C. microcephala* compared to the other species. Interestingly, a decrease in the concentration of the BE was observed during the last 1-2 samplings and at the same time an increase of the concentration of the SE was observed. Very little BE was extracted by warm water treatment from *A. minutissima*, *C. minuta* and in *C. vulgate*, whereas it was comparable with the values from (de Brouwer & Stal, 2002; Staats et al., 1999).

All the other genera (except *Pseudostauroopsis*) in axenic state showed the absence of EPS structures like stalks, capsules or tubes or the presence of high amounts of warm water extractable EPS, which was reflected in a lower BE content in all isolates. However, in non-axenic conditions, *C. microcephala* (D-23) showed the presence of tube and capsule like structures and isolates of *A. minutissima* showed the presence of capsules (Chapter 5) which were not observed under axenic conditions. Here, none of the isolate showed very firm attachment to the bottom of the flasks. Just shaking was found to be useful for the resuspension of the cells. Thus, bacteria might be playing an important role in cell adhesion (Cole, 1982) (for a discussion about diatom-bacteria interactions, see Chapter 8).

Isolates of *Pseudostauroopsis* show the presence EPS structures such as intercellular pads (Hoagland et al., 1993), which hold adjacent cells together to form ribbon-like colonies. The temperature of the water used for extraction of the BE was insufficient for dissolution of such pads and therefore might be the reason for finding such high contents of carbohydrates in the RC fraction. These pads can be only extracted by using very harsh treatment such as heating in NaHCO<sub>3</sub> at 95 °C (Chapter 7).

All our isolates demonstrated relatively similar amounts of carbohydrates in the RC fractions. This fraction mainly consists of structural carbohydrates (de Brouwer & Stal, 2002) and carbohydrates involved in the formation of silicious walls of diatoms (Hecky et al., 1973) or in species like *Pseudostauroopsis*, part of it might contain intercellular pads, which cannot dissolve in warm water treatment (Chapter 7).

The SE showed genus-specific signatures of monosaccharides. In all *Cymbella* species, a very high amount of galactose was found. In *A. minutissima*, the three sugars galactose, fucose and mannose/xylose contributed to the same extent, whereas

in *Pseudostauroopsis* mannose/xylose was the abundant sugar (Fig. 3). In all isolates, the only studied uronic acid, N-acetyl-glucosamine was present in very small amounts (3-5%) when compared with the reported 33-44% for *Cylindrotheca closterium* (de Brouwer & Stal, 2002). Thus are no reports for the direct comparison of sugar components in the SE of freshwater diatom genera that we studied. The genera, which have been studied, are marine e.g. the presence of a relatively high amount of galactose in the SE fraction of marine diatoms *C. closterium* and *Nitzschia* sp has been reported. However, in another study, higher amount of xylose in *C. closterium* and glucose in *Navicula salinarum* have been reported (Staats et al., 1999).

In PCA, the monosaccharide profiles of all the isolates grouped mostly according to their taxonomy (i.e. two isolates of *Pseudostauroopsis*, four isolates of *C. microcephala*) in most of the cases. Interestingly, one isolate of *A. minutissima* was grouped with the pore water from biofilm. As many biofilms are dominated by *A. minutissima* (Chapters 2 and 3) influence of it on pore water can be justified.

To conclude, 1. We found that there were species-specific signatures in the monosaccharide composition of the soluble EPS, if cultivated under identical conditions. Thus, analysis of EPS might be an additional tool for confirming phylogenies. Further investigations are needed in this aspect.

2. We could analyse the diatom genera, which produced a relatively high amounts of EPS among the dominant cultivated members, which are likely to contribute to the pore water EPS in biofilms and supply an important carbon source for bacteria.

3. Dominant species like *A. minutissima* might influence the monosaccharide profile of the biofilms as revealed by PCA.

**Acknowledgements**

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## Chapter 7

The complex extracellular polysaccharide of various diatom species from epilithic biofilms (Lake Constance, Germany)

Rahul A Bahulikar and Peter Kroth

Faculty of Biology, University of Konstanz, University str. 10, Konstanz Germany

Key words: epilithic biofilm, bacterial abundance, diatom diversity, EPS,

**Abstract**

This study is focused on the characterization of extracellular polymeric substances (EPS) from a total of 14 diatoms belonging to six genera namely *Achnanthes*, *Cymbella*, *Fragilaria*, *Punctastriata*, *Staurosira* and *Pseudostaurosira* which were isolated from epilithic biofilms of the littoral zone of Lake Constance. In all isolates, frustule-associated EPS was observed. In addition, several isolates showed the presence of chain-like colonies, where cell-cell attachment is mediated by intercellular pads to maintain colony integrity, EPS from all isolates was extracted by a sequential extraction procedure resulted in five different fractions. The monosaccharide composition of each fraction was analysed by HPLC. Most of the intercellular pads were dissolved by hot bicarbonate, whereas the frustules dissolved by hot alkali treatment. FITC labelled-lectins proved to be useful for the localization of various sugar moieties and comparison of the data obtained after chemical extraction.

## Introduction

Diatoms are pioneering eukaryotic organisms that initiate biofilm formation on any new surface when immersed in the water. They are well known producers of copious amounts of extracellular polymeric substance (EPS) (Hoagland et al., 1993). EPS helps or attracts other organisms to colonize on the substratum. Then the subsequent growth of the diatoms and the other colonizing organisms finally lead to a complex biofilm formation (Jackson et al., 2001). Diatoms are important primary producers and dominant members in biofilms of the littoral zone (Ács et al., 2000; Buczkó & Ács, 1997; Chan et al., 2003).

We used both raphid and araphid diatoms in our study. The araphid diatoms such as *Fragilaria*, *Pseudostauroopsis*, *Staurosira* and *Punctastriata* display chain-like colonies. These colonies are formed by cell-cell attachments via spines originating from the frustules, which interlock two adjacent cells (Williams & Round, 1987)(Fig. 2, chapter 4). Here, EPS works as a cementing material. Another way to keep cells together is by formation of intercellular pads composed of EPS as observed in *F. capucina*. Such EPS pads are ecologically important for dispersal and population development of raphid diatoms. In our study, *Achnanthes minutissima*, *Cymbella microcephala* and *C. minuta* did not show the presence of specialized EPS structures when they were axenic. However, a thin film of frustule associated EPS (FAE) is always present in these diatoms (Chapters 4 and 6). Among the diatoms which are used in the present study, *A. minutissima* is a cosmopolitan species of small size with a very broad ecological magnitude and dominance in biofilms (Ács et al., 2003; Barbiero, 2000). At our study site, this diatom was found to be present in high numbers throughout the year. *C. microcephala* and *C. minuta* are also small diatoms found to be dominant in epilithic biofilms of various lakes (Barbiero, 2000) including the littoral zone of Lake Constance (Chapter 2 and Chapter 3).

To our knowledge there are no reports available on the sequential extraction and analysis of EPS secreted by the diatoms mentioned above. Isolation and fractionation of EPS from unique species requires the optimization of extraction procedures and fractionation protocols according to the chemistry and location of the polysaccharides (Chiovitti et al., 2003). Here, efforts have been taken for extraction, fractionation and analysis of the EPS from various raphid and araphid diatoms found at our study site.

Various published methods were tried to optimize a protocol which was then successfully used for EPS fractionation from various diatoms. The monosaccharide profiles of all the fractions of a single isolate were compared with the other diatoms. Here lectins were also used for EPS localization. Lectins are proteins of non-immune origin and they bind to sugars specifically and reversibly (Lis & Sharon, 1986). When labelled with fluorescent dyes lectins become a useful tool for detection of sugars and have been used before in the localization of EPS. The localization approach was used for the comparison of the data obtained by chemical extraction.

## Material and Methods

### *Isolation of organisms*

All studied species (Table 1) were isolated from epilithic biofilms of the littoral zone of Lake Constance (Germany, 47°41'N, 9°11'E). The cultures were maintained in diatom medium (DM) (Watanabe, 2005) at 16 °C for 16 hr, 50 µE illumination was provided by cool-white fluorescent tubes. Isolated strains were streaked on DM plates with a combination of three antibiotics (10 µg/ml kanamycin, 10 µg/ml amikacin and 10 µg/ml erythromycin) to get rid of bacterial contaminations. Clean axenic colonies were picked microscopically. The cultures were checked for bacterial contamination by epifluorescence microscopy, after staining them with SYBR Green I (Ambrex Bioscience, Germany) and streaking them on nutrient agar plates.

### *Experimental design*

All axenic isolates (Table 1) were grown in either 5 x 1 l flasks containing 600 ml or 1 x 5 l flask containing 3 l of DM with aeration or the flasks were shaken once a day. Cultures were harvested at the stationary phase (24<sup>th</sup> day after inoculation) 1 ml was used for measuring the concentration of soluble EPS and for measuring the chlorophyll *a* content to determine the diatom growth (as described in Chapter 6). The cell pellets were used for extraction of different carbohydrate fractions as described below.

### *Isolation and analysis of carbohydrate fractions*

For preliminary experiments, half of the cell pellets were divided in various equal parts and each part was resuspended in the following solutions 1) 0.5 M NaOH, 2) 0.1 M NaOH, 3) 1.0 M NaCl, 4) 0.5 M NaCl, 5) 0.1 M EDTA pH 7.0, 6) 0.05 M EDTA, 7) 0.1 M EDTA pH 8.0, 8) 0.05 M EDTA, 9) 0.5 M NaHCO<sub>3</sub> and 10) 1 M NaHCO<sub>3</sub> and were incubated at two different temperatures (20 °C and 30 °C). The remaining cell pellets were then defatted as described below, treated with the same solutions (mentioned above) and incubated at 95°C for 1 h. Before and after each treatment the EPS extraction was monitored staining with alcian blue (1% w/v in 3% acetic acid) (Daniel et al., 1987).

After various trial experiments following fractionation procedure was applied:

Cells were harvested at stationary phase and cell pellets were immediately treated for EPS extraction to avoid any possible storage effect.

Fraction 1: The cultures were centrifuged at 5100 rpm for 10 min and the spent medium was concentrated using a rotary evaporator to 1/10<sup>th</sup> of its original volume and was precipitated by 5 volumes of alcohol followed by overnight incubation at -20 °C. Then it was centrifuged and the resultant EPS pellet was subsequently air dried or dried under flow of nitrogen. This fraction was named as SE (Soluble EPS) (Abdullahi et al., 2006; Bellinger et al., 2005; Chiovitti et al., 2003; de Brouwer & Stal, 2002).

Fraction 2: Resultant cells pellets were incubated in 10 volumes of distilled water at 30 °C for 1 h and followed by centrifugation (WW-warm water soluble EPS) (de Brouwer & Stal, 2002; Staats et al., 1999). Supernatant was precipitated and processed as mentioned above.

Fraction 3: Cell pellets from Fraction 2 were washed 4-5 times with 90% ethanol for 10-15 min at room temperature then centrifuged at 5100 rpm for 10 min after each extraction. The defatted cell pellets were washed twice with distilled water followed by incubation at 90 °C in distilled water and this fraction was termed as hot water soluble EPS (HW) (Wustman et al., 1997).

Fraction 4: The pellets were extracted twice with 0.5 M NaHCO<sub>3</sub> at 95 °C and this was termed as hot bicarbonate (HB) fraction (Abdullahi et al., 2006; Bellinger et al., 2005; Chiovitti et al., 2003; Wustman et al., 1997).

Fraction 5: The final extraction was done with 1 M NaOH with 0.2 M NaBH<sub>4</sub> was added to the cell pellets and incubated at 95 °C for 1h (Hot alkali - HA) (Abdullahi et al., 2006; Chiovitti et al., 2003).

Last three fractions (HW, HB and HA) were dialysed overnight (6-8 kd cut off) against distilled water and were freeze-dried (Abdullahi et al., 2006; Wustman et al., 1997).

Carbohydrate concentrations were measured using phenol / H<sub>2</sub>SO<sub>4</sub> assay (Dubois et al., 1956) using glucose as the standard. Bicinchoninic acid assay was used for analysing protein concentration from HW, HB and HA fractions by following manufacturers protocol (Pierce, Germany) and using BSA as the standard.

### *Monosaccharide composition*

EPS pellets from all five fractions SE, WW, HW, HB and HA were used for hydrolysis and subsequent monosaccharide analysis. 5 mg of dried polysaccharide was hydrolyzed to monosaccharides by heating with 2M Tri-FluoroAcetic acid (30 min at 121 °C). The monosaccharides composition was analyzed by HPLC equipped

with a CarboPac PA10 column (Dionex, Germany) and a pulse amperometric detector (Jahnel et al., 1998) (Dionex, Germany). “Chameleon” software (Dionex, Germany) was used to analyze the individual runs.

For quantification, we used commercially available 8 monosaccharides namely, fucose (Fuc), galactose (Gal), glucose (Glc), rhamnose (Rha), fructose (Fru), arabinose (Ara), ribose (Rib), Mannose or Xylose and one uronic acid N-acetylglucosamine (GlcNAc) as the standards. Mannose and xylose were co-eluted in HPLC; therefore, we could not distinguish between them and has been mentioned Man/Xyl as the values represent either one or both of the sugars.

### *Lectin labelling*

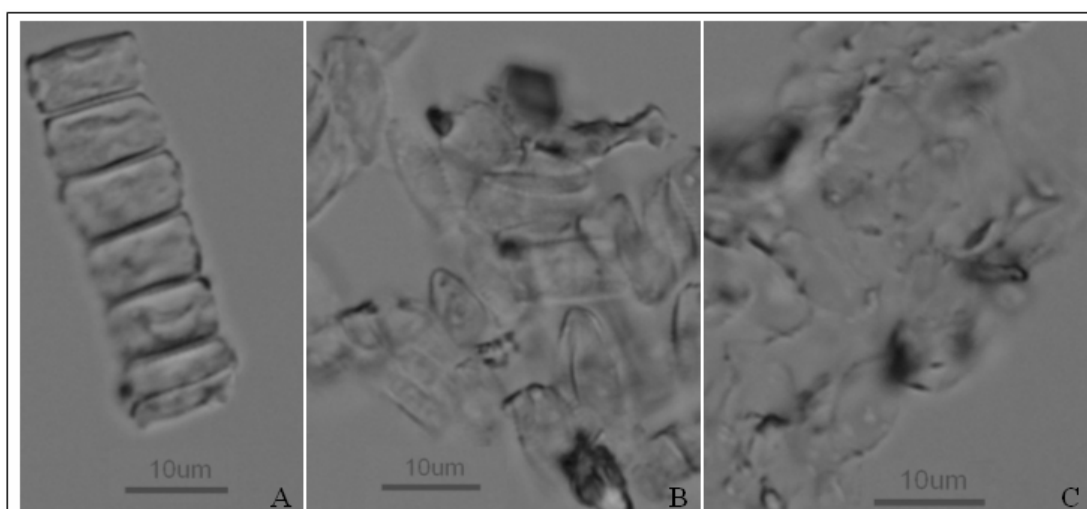
Six FITC labelled-lectins and DAPI were used for localization of EPS. Procedure and abbreviations used are as per in Chapter 5.

### *DATA analysis*

Principal component analysis (PCA) of monosaccharide profiles from all fractions of all isolates was done using MVSP software (Kovach, 2002).

## Results

In this report we tested the utility of previously reported solutions (Abdullahi et al., 2006; Bhosale et al., 1993; Chiovitti et al., 2003; Staats et al., 1999; Wustman et al., 1997) for extraction of intercellular EPS pads and frustule associated EPS (FAE) from various diatom isolates mentioned in Table 1. Before and after each treatment, EPS extraction was monitored by staining with alcian blue. In addition to the reported concentrations, one higher and /or one lower concentration were also tested and preliminary experiments were performed at 20 °C and 30 °C accordingly. We could extract neither pads nor FAE at 20 °C or 30 °C using any of the solutions. Cell leakage was noted at both concentrations of NaOH. At all used concentrations of NaCl and EDTA intercellular pads were not dissolved even at higher temperatures. However, they partially/totally dissolved in concentrations of 0.5 M and 1.0 M NaHCO<sub>3</sub> when incubated at 95 °C for 1 h. At used concentrations of NaOH intercellular pads as well as almost all frustules were dissolved.



**Fig. 1** Effect of sequential extraction procedure on colony structure of diatom *Pseudostaurosira* (I-01) A. Defatted colonies treated with hot water B. intercellular adhesion was removed by hot bicarbonate treatment and C. Most of the frustules were dissolved after hot alkali treatment

From all the preliminary experiments, we found that

1. Lower amounts of carbohydrates were present in the spent medium of most of the diatoms than *C. microcephala* (isolate B-08) where it was 90 µg/ml.
2. Incubation of the cell pellets in distilled water at 30 °C yielded small amounts of probably loosely attached EPS.

3. High amounts of EPS were observed in the hot bicarbonate fraction where most of the intercellular pads were dissolved (Table 1).
4. All FAE, pads and frustules were dissolved in the hot alkali treatment.

Considering all these observations we used a modified extraction scheme which included saving of spent medium for soluble EPS analysis (SE), followed by sequential extraction of cell pellet at 30 °C (WW), 90 °C in distilled water (HW), hot bicarbonate (HB) and finally hot alkali treatment (HA).

Table 1: Protein and carbohydrate content present in from various fractions of each diatom. All values are in mg/g of fresh weight of the cell pellet.

Isolate no.	Name of isolate	Proteins				EPS			
		HW	HB	HA	Total	HW	HB	HA	Total
A-06	<i>Fragilaria capucina</i>	1.70	23.42	5.87	30.98	3.43	7.27	1.24	11.94
Dx7	<i>Pseudostauropsis</i>	0.75	6.67	9.14	16.56	1.65	3.00	2.35	6.99
F-02	<i>Pseudostauropsis</i>	0.10	0.40	2.26	2.76	3.76	5.55	5.28	14.59
F-03	<i>Pseudostauropsis</i>	ND	1.88	1.95	3.83	2.26	4.61	2.32	9.19
F-05	<i>Pseudostauropsis</i>	0.49	0.05	1.59	2.12	1.09	1.84	2.75	5.68
I-01	<i>Pseudostauropsis</i>	1.48	1.20	2.05	4.74	3.43	1.71	0.68	5.82
I-23	<i>Pseudostauropsis</i>	1.09	0.83	3.84	5.76	1.33	8.00	1.67	11.00
I-61	<i>Pseudostauropsis</i>	0.60	0.73	4.67	6.00	1.60	2.33	9.48	13.41
C-07	<i>Punctastriata</i>	1.88	3.36	1.65	6.89	2.17	2.92	3.90	8.98
E-05	<i>Punctastriata</i>	0.14	0.21	2.98	3.33	0.88	1.29	2.06	4.23
C-05	<i>Staurosira</i>	0.93	7.64	1.10	9.67	0.38	5.47	2.24	8.09
I-141	<i>Staurosira</i>	0.60	0.73	4.67	6.00	1.60	2.33	9.48	13.41
I-117	<i>Achnanthes minutissima</i>	0.21	0.39	2.58	3.18	0.45	0.62	0.87	1.94
B-08	<i>Cymbella microcephala</i>	3.69	5.26	0.28	9.22	3.68	6.07	0.96	10.71
I-51	<i>Cymbella minuta</i>	0.72	5.90	2.55	9.17	2.84	6.22	0.94	10.00

HW; hot water, HB; hot carbonate and HA; hot alkaline fractions, ND; not detectable

We observed different amounts of protein in the last three fractions (HW, HB and HA (In first two fractions proteins were not detectable)), whereas high concentrations of proteins were observed in isolate *F. capucina* (A-6) (Table 1).

Fig. 2A

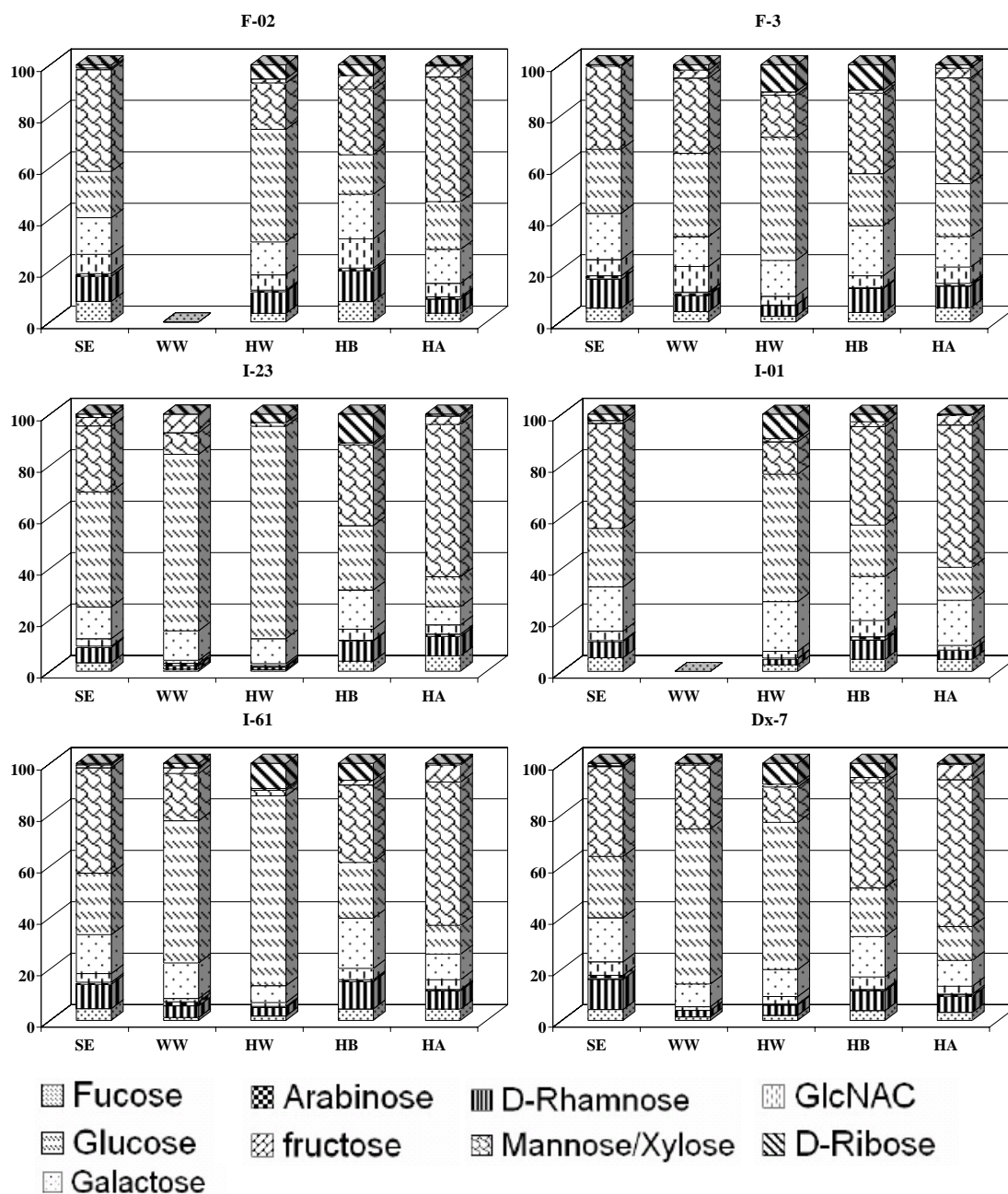
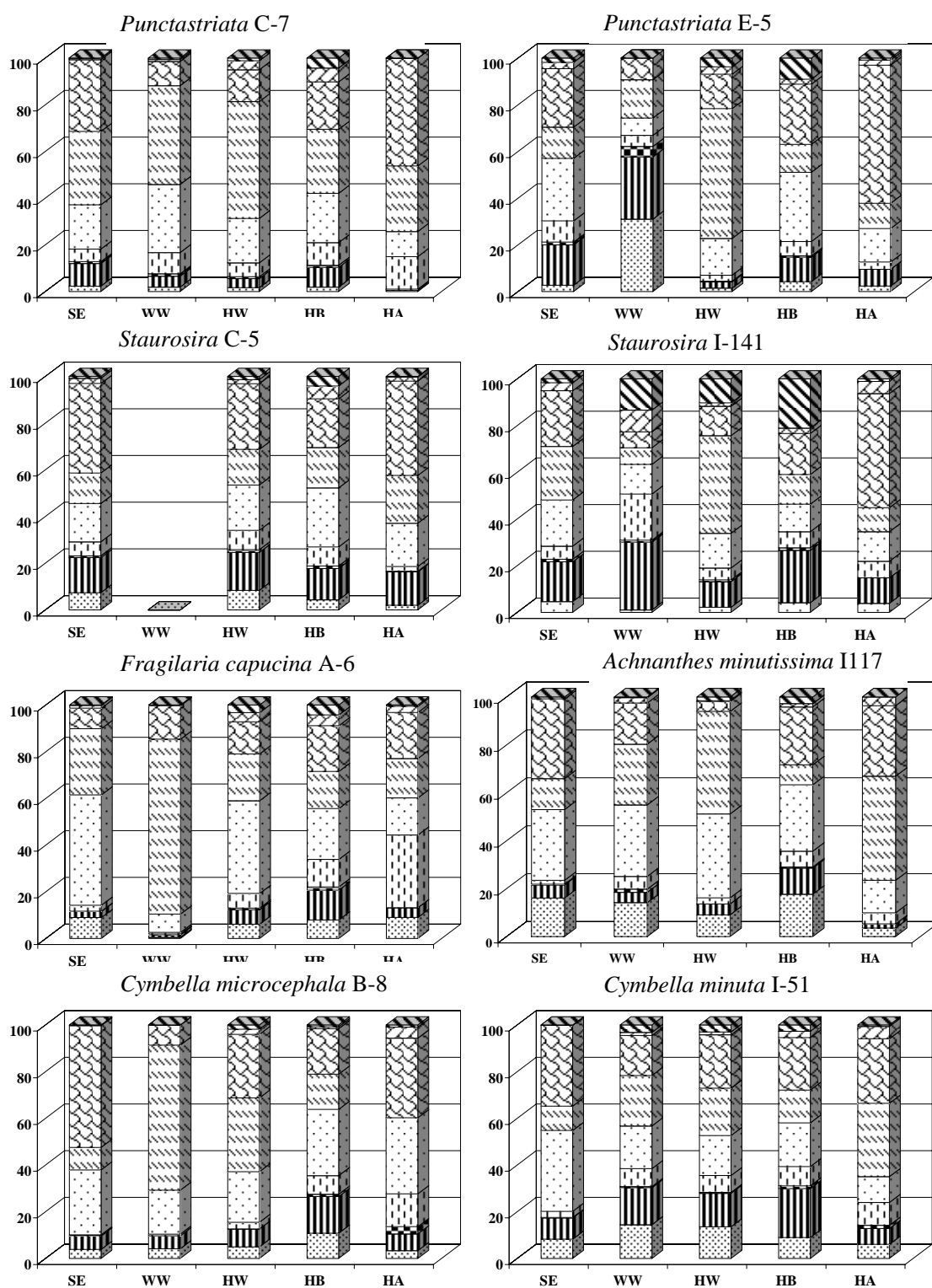


Fig. 2 Monosaccharide profiles of all fractions from various isolates A. various isolates of *Pseudostauropsis* and B. isolates of *Staurosira*, *Fragilaria capucina*, *Punctastrita*, *Achnanthes minutissima*, *Cymbella minuta* and *C. microcephala*. SE: soluble EPS, WW: warm water soluble EPS, HW: hot water soluble EPS, HB: hot bicarbonate soluble EPS and HA: hot alkali soluble EPS.

Fig. 2B



Significant similarity was observed in the monosaccharide composition between all the EPS fractions of a single isolate, whereas phylogenetically related isolates of single species when compared for monosaccharide composition in individual components showed similarities in most of the cases.

*Carbohydrate composition*

The monosaccharide composition of all the EPS fractions from the isolates is summarized in Fig 1. Significant differences were observed in the relative percentage of sugars in all the fractions of a single isolate. Except in *C. minuta*, three fractions (WW, HW and HB) showed similar monosaccharide profiles. FITC labelled-lectins were useful to detect the presence of specific sugar moieties in pads and / or FAE. DAPI and two lectin (Con A and HAA) bound to the pads and FAE of nearly all isolates, whereas variable binding was observed by other lectins. No species-specific binding pattern was observed. Table 2 gives information about lectin binding to FAE and /or to the intercellular pads.

*Composition of EPS in various fractions of the isolates*

*A) Araphid diatoms*

*Pseudostauropsis* species

In the six isolates studied, the respective fractions were similar in their sugar composition. SE and HB fractions were highly heterogeneous, whereas in HW and WW high amounts of Glc and in HA Man/Xyl were found (Fig. 2A). High variation of lectin binding was observed in various isolates of *Pseudostauropsis*, whereas DAPI epifluorescence was observed in all isolates (Table 2A)

*Fragilaria capucina*

All fractions of *F. capucina* (A-06) showed high differences in the composition of sugar monomers. SE and HW fractions contained high amounts of Gal and then Glc, however, the WW fraction was dominated by Glc only. HB and HA fractions showed the presence of Gal, Glc and Man/Xyl. Interestingly, sequential increases in concentrations of GlcNAc were observed starting from low concentrations in the WW fraction to high concentration was in HA fraction, whereas the amount of Gal showed exactly opposite pattern. In all fractions, the other sugars were present in smaller quantities or in traces. This was the only HA fraction in all the studied isolates where the concentration of Man/Xyl sugar was the lowest (19.69%, Fig 2B). Five lectins (except UEA) bound to the intercellular pads as well as to the frustule, whereas no epifluorescence was observed to intercellular pads by DAPI (Table 2), indicating the presence of Glc, Man, GalNAc and GlcNAc

Table 2: Lectin labelling

Isolate No.	Part	Con A	WGA	PSA	HAA	LEA	UEA	DAPI
A-06	Frustule	+	+	+	+	±	-	+
	Cell-Cell	+	+	+	+	±	-	-
Dx-07	Frustule	+	+	+	+	+	+	+
	Cell-Cell	+	+	+	+	+	+	+
F-02	Frustule	+	+	+	+	+	-	+
	Cell-Cell	+	-	-	+	-	-	+
F-03	Frustule	+	+	+	+	+	-	+
	Cell-Cell	+	-	+	+	+	-	+
F-05	Frustule	+	±	+	+	+	-	+
	Cell-Cell	-	-	+	+	±	-	+
I-01	Frustule	+	+	+	+	+	-	+
	Cell-Cell	+	+	+	+	+	-	+
I-23	Frustule	+	±	+	+	±	-	+
	Cell-Cell	+	±	+	+	-	-	±
I-61	Frustule	+	+	+	+	+	-	+
	Cell-Cell	+	-	-	+	-	-	+
C-07	Frustule	+	+	+	+	±	-	+
	Cell-Cell	+	-	+	+	-	-	+
E-05	Frustule	+	±	+	+	±	-	+
	Cell-Cell	+	±	+	+	-	-	+
C-05	Frustule	+	+	+	+	+	+	+
	Cell-Cell	+	+	+	+	+	+	+
I-141	Frustule	+	+	+	+	+	+	+
	Cell-Cell	+	+	+	+	+	+	+

+ Staining observed, - No staining observed, +/- variable

### *Staurosira* species

SE fractions of *Staurosira* (C5 and I141) showed different monomer compositions. In SE of C-5, Man/Xyl were the dominant sugar(s), whereas in I-141 Glc and Man/Xyl were equally distributed. WW fraction of I-141 was enriched in

Rha; unfortunately this fraction was not available in C-5 for comparison. The HB fraction revealed abundant amount of Rha and Rib in I-141, whereas Gal, Glc and Man/Xyl were abundant in C-5. The HA fraction of both isolates showed very high concentrations of Man/Xyl (Fig. 2B). Binding of all used lectins and DAPI epifluorescence was observed to the FAE and to the intercellular pads (Table 2).

#### *Punctastriata* species

SE and HB fractions of the two isolates of *Punctastriata* sp (C-7 and E-5) were very heterogeneous with no dominant sugar monomers. The WW fraction in both isolates showed very high differences with respect to their monosaccharide composition. In C-7, Gal (42.31%) was the dominant sugar whereas E-5 showed the dominance of Fuc (26.66%) and Rha (31.01%). Both isolates showed high amount of Glc in HW, whereas, Man/Xyl were the dominant sugar(s) in HA of both isolates (Fig 2B). Con A, PSA and HAA binding to pads and FAE of both isolates was observed. However, no UAE labelling observed to either to the frustules or to the pads. Interestingly, LEA binding was detected only to the frustules of both isolates (Table 2).

#### B) *Raphid diatoms*

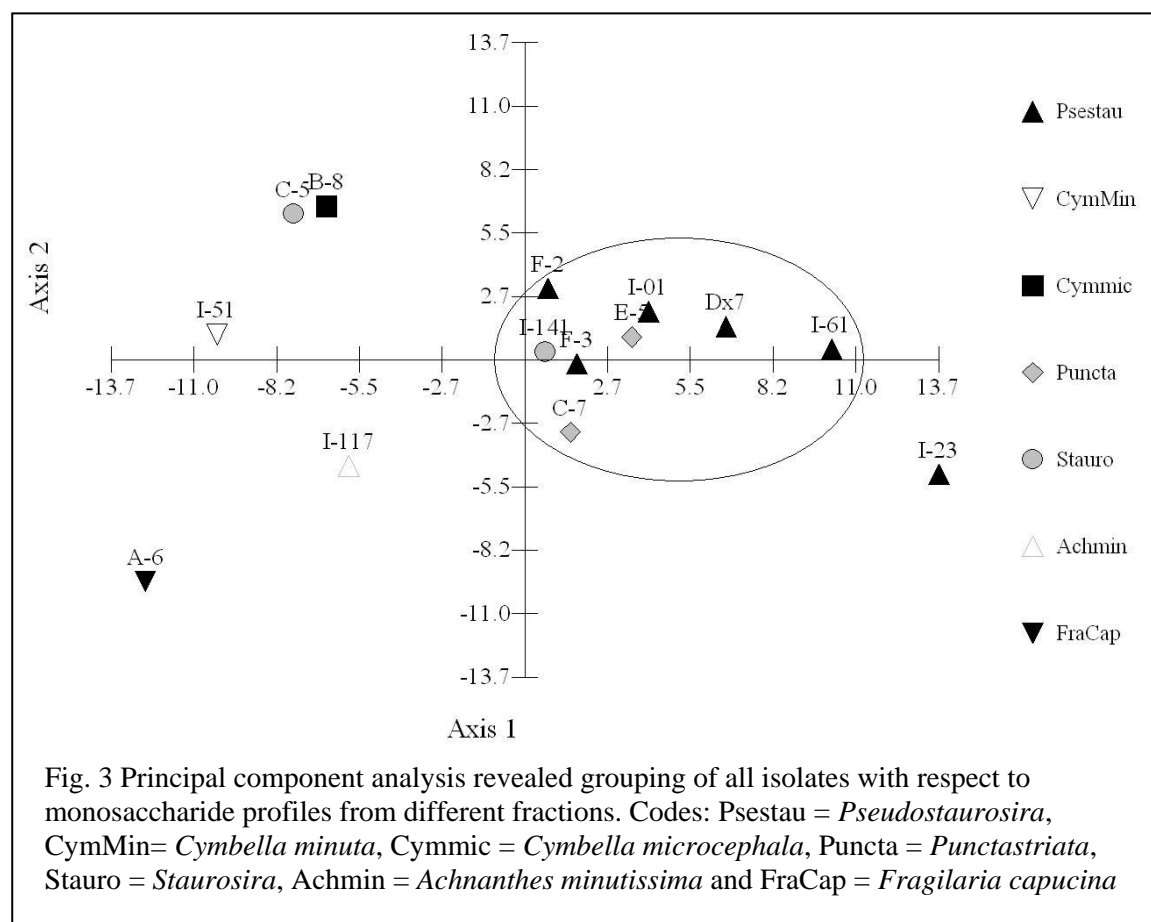
*Achnanthes minutissima*, *Cymbella microcephala* and *C. minuta* do not show EPS structures like tubes, pads or stalks. The SE fraction of *A. minutissima* (I-117) was enriched with Man/Xyl, WW and HB with Gal and HW and HA fraction with Glc.

The SE and WW fractions of *Cymbella microcephala* (B-8) were dominated by Man/Xyl (52%) and Glc (62%), respectively. HW, HB and HA fractions were highly heterogeneous with increasing amounts of GlcNAc (2.68, 7.98 and 14.06%) (Fig. 2B).

Interestingly, in *C. minuta* (I-51) and *C. microcephala* (B-08), the SE fractions were relatively similar in sugar composition and the HA fraction of *C. minuta* showed a dominance of Glc. The remaining three fractions showed nearly identical patterns (Fig 2B).

The lectin binding profile for frustules of *A. minutissima* and *C. microcephala* were identical as reported before for the frustules of non-axenic stains (Chapter 4).

## Principal coordinate analysis (PCA)



PCA is a data reduction method and it facilitates the multidimensional data to smaller number of dimensions with a minimum loss of information. Hence it was used to investigate correlation of sugar profiles from all the fractions of a single isolate with all the fractions of other isolates (except WW data due to unavailability of complete dataset). Most of the isolates from *Pseudostaurosira* (Except I-23), *Staurosira* (Except C-5) and *Punctastriata* were grouped together demonstrating a similarity of sugar profiles. However, *F. capucina* showed very high differences from them and was out-grouped. Because of unknown reason *C. microcephala* (B-8) and one isolate from *Staurosira* (C-5) grouped together. The monosaccharide profiles of *C. minuta* and *A. minutissima* were also clearly separated from profiles of other diatoms (Fig. 3).

## Discussion

Here we report about sequential chemical extractions of EPS and its subsequent monosaccharide analyses from various diatoms isolated from epilithic biofilms of Lake Constance. Labelling of carbohydrates by lectins was used for comparison with the extracted composition of sugars with the in situ sugars. All the studied diatoms species were among dominant members present in the epilithic biofilms of Lake Constance (Chapter 2 and Chapter 3).

EPS extraction procedures were reported previously for *Cylindrotheca closterium* and *Navicula salinarum* using EDTA, NaCl and NaOH at lower temperatures (Staats et al., 1999). In our study, these approaches were found neither to be useful for the extraction of intercellular pads, nor for extraction of FAE. Incubation of cells at two different lower temperatures in two concentrations of NaOH, it resulted in the cell leakage. This observation is in accordance with Staats et al. (1999). NaOH was found to be useful at high temperatures. Most of the FAE and most of the intercellular pads were dissolved in hot bicarbonate at 95 °C (HB fraction) and a total dissolution of diatom frustules of all studied species was observed in hot alkali (HA), which has been reported previously (Abdullahi et al., 2006; Chiovitti et al., 2003).

### *Comparison of monosaccharides in individual fractions*

#### *Soluble EPS (SE)*

Relatively high amounts of SE was observed in the spent medium of *C. microcephala* (B8) (~70 mg l<sup>-1</sup>), whereas in other isolates the SE content was either very low or not detectable. Lower concentrations of SE was also reported for *Pinnularia viridis* (Chiovitti et al., 2003), *C. closterium* and *N. salinarum* (Staats et al., 1999). Monosaccharide composition of SE fractions in *A. minutissima* (I-117), *C. microcephala* (B-08), *C. minuta* (I-51), and *Pseudostarosira* (Dx7, F-03) were similar as reported before (Chapter 6). All *Pseudostauropsis* isolates showed similar pattern of monosaccharides within every fraction. Both isolates of *Staurosira* and *Punctastriata* were heterogeneous, whereas very high concentration of Glc was observed in the *F. capucina* (A-06). Monosaccharide composition of SE showed species and genera specific patterns (Chapter 6).

#### *Warm water soluble EPS (WW)*

Loosely attached bound EPS can be extracted by warm water (WW) treatment (de Brouwer & Stal, 2002; Staats et al., 1999). This method was used by many researchers for extraction of warm water soluble polysaccharides from sediments, biofilms and from axenic cultures (de Brouwer & Stal, 2002; Staats et al., 1999; Stal, 2003). During this treatment, there is a possibility of a contamination by internal storage carbohydrates (Chiovitti et al., 2004). The intracellular glucans have a smaller size (from 4-13 kd) while EPS is usually larger than 13 kd (Bhosale et al., 1995). For separation of intracellular carbohydrates and extracellular polymers we therefore used another method, i.e. precipitation of the WW fraction in cold ethanol (-20°C and 75% final concentration). It has been shown that the precipitated polysaccharides (>100 kd) obtained are free of contamination by intracellular sugars (de Brouwer & Stal, 2002; de Brouwer & Stal, 2004).

WW treatment in some isolates yielded very little or no polysaccharide, therefore in some cases WW data is not available. Glc rich bound EPS have been reported from *Cylindrotheca closterium*, *Nitzschia* (de Brouwer & Stal, 2002), *C. closterium*, *Navicula salinarum* (Staats et al., 1999) and *P. viridis* (Chiovitti et al., 2003). In accordance, in our study Glc was the dominant (40-60%) sugar in isolates of *Pseudostauropsis*, *F. capucina*, one isolate of *Punctastriata* (C-7) and *C. microcephala* (B-8), whereas in the remaining isolates the WW fraction was very heterogeneous. Therefore, monosaccharide profiles might be different in different species.

### *Hot water soluble EPS (HW)*

Alcian blue staining demonstrated the presence of polysaccharides on the surface of the frustules, whereas the intercellular pads were still intact even after WW extraction. Therefore, the cells were defatted and the cellular contents were removed before further treatments. The defatting procedure basically removes cellular contents therefore contamination of the intracellular carbohydrates in the EPS fractions can be avoided. After defatting, hot water soluble material was extracted by incubating the cell pellet in distilled water at 90 °C (HW) (Wustman et al., 1997). Differences were observed in the sugar composition between WW and HW fractions from a particular diatom. In nearly all *Pseudostauropsira*, *Punctastriata* isolates and in *A. minutissima*, Glc concentrations were higher in HW than WW, whereas in remaining isolates it was less (except *C. minuta*, similar Glc content was observed). Higher amounts of Glc was demonstrated in various diatom species (Bellinger et al., 2005; Chiovitti et al., 2004)

and Chiovitti et al. (2004) suspected that the glucose might be of intracellular origin. Thus it was unclear what was exactly extracted by this procedure.

*Hot bicarbonate soluble EPS (HB)*

Extraction of EPS with sodium bicarbonate was performed at 95 °C. This procedure is useful to solubilize the relatively gelatinous EPS fraction (Chiovitti et al., 2003) which has been reported to be rather heterogeneous and with lower amount of Glc than other fractions (Chiovitti et al., 2003; Wustman et al., 1997). In accordance with this, all our diatom isolates showed a heterogeneous monosaccharide profiles and also Glc was reduced noticeably compared to HW. After this treatment, only small numbers of intact diatom chains were observed, therefore almost all EPS responsible for cell-cell attachment might have been dissolved. This is in accordance with the previous report (Wustman et al., 1997), where dissolution of EPS structures such as stalks and capsules from various diatoms was observed when treated with hot carbonate.

*Hot alkali soluble EPS (HA)*

After HB treatment mostly the FAE fraction remained. HA treatment is basically used for the extraction of frustule associated EPS (Chiovitti et al., 2005). In our study, frustules of all the isolates were extracted in this fraction. Monosaccharide profiles showed the presence of high Man/Xyl in various isolates. In *Pseudostauropsis*, two isolates (F-2 and F-3) showed 40-41% of Man/Xyl and in remaining isolates it was 55-59%. According to earlier reports, the Man is probably associated with silicified frustules and removal of it might be the cause of frustule dissolution (Chiovitti et al., 2005). GlcNAc has been also found in the HA fraction (Chiovitti et al., 2005; Chiovitti et al., 2003). As Man/Xyl were undistinguishable have lectin labelling was useful in this case where the abundance of Man instead of Xyl was indicated by labelling with the PSA lectin, which bound to the frustules. According, to Brett & Waldron (1990) hot alkaline treatment disrupts the hydrogen bonding and results in the dissolution of polysaccharides associated with the frustules.

*EPS Localization with lectins*

Localization of EPS by FITC labelled-lectins was found to be useful to complement the results obtained after the HPLC analysis. HAA is specific for GalNAc and intense labelling was observed in all chain forming species. We also found intense binding of HAA, Con A (Glc and Man), PSA (Man) to the intracellular pads demonstrating the presence of higher amount of GalNAc, Glc and Man in the

pads whereas, the lectin LEA (is specific for GlcNAc) did not bound very well and was conformed by HPLC analyses. As Man and Xyl co-eluted it was not possible to detect exact amount of each sugar. PSA lectin therefore was useful to detect presence of the Man in the FAE and pads of most of the chain forming isolates. Higher fluorescence intensity of the intercellular pads was observed in comparison to the FAE therefore we suspected higher amounts of Man in the pads than FAE. Fuc was present in lower amounts in nearly all fractions as shown by HPLC and was conformed by lectin labelling. The lectin UAE specifically binds to the Fuc (UAE) and it showed faint or no binding to either FAE or to the pads. Various sugars were detected in all fractions of *A. minutissima* and *C. microcephala* and were at different concentrations but because of unknown reasons only binding of Con A and DAPI to the frustules was observed (Chapter 4).

### *Proteins in different fractions*

High amounts of proteins have been previously demonstrated within the HB fraction of *P. viridis* (Chiovitti et al., 2003). In our study, *F. capucina* (A6) showed the presence of high amount of proteins in the HB fraction. However, *Pseudostauroopsis*, *Staurosira* and *Punctastriata* demonstrated different amounts of proteins in various fractions. This observation was in accordance with by Hoagland et al. (1993). The presence of proteoglycans was reported in gliding trails of *Craspedosaurus australis* by using antibody localization (Lind et al., 1997) and glycoproteins (Chiovitti et al., 2005). Proteins were isolated and characterized from cell walls of *Cylindrotheca fusiformis*, and those proteins might involved in morphogenesis (Kröger et al., 1997) and as silica precipitating peptides (Kröger et al., 2001). Therefore proteins might be important in formation of frustules and intercellular pads.

### *Structure of intercellular pads*

In our study, all chain-forming diatoms were araphids where cell-cell attachment is mediated by the intercellular EPS pads. Extraction with hot bicarbonate resulted in reduction of the chain length from 7-9 to 3-4 cells per colony. This suggests dissolution of most of the intercellular EPS. HB fraction contains high amounts of proteins, carbohydrates and uronic acids. The monosaccharide composition showed a variable amount of GlcNAc and lectins indicated presence of GalNAc (HAA) and also GlcNAc (WGA and LEA) in the intercellular pads. Uronic acids are known form a junction zone/ intermolecular bridges via divalent cations affecting polysaccharide solubility (Chiovitti et al., 2003). According to Hoagland et

al. (1993), pads are 5-10 times stronger than stalks and contain internal and external cores which can be stained by periodic acid-schiff reaction (Daniel et al., 1987). From our study, we suspect that such two core structures might generally be present in the pads of chain forming diatoms. Extraction with hot bicarbonate might have removed the outer core because the some short-chained colonies remained. Extraction with hot alkali showed the dissolution of frustule along with inner core of the pads.

### *PCA and phylogeny*

PCA was used for the comparison of monosaccharide profiles of each individual fraction of a isolate with the profiles of representative fraction of in all other individuals. Differences in monosaccharide profiles were observed in all different fractions of the single diatom isolates and related diatoms showed similar monosaccharide signatures in the respective fractions (e.g. the HA fractions of all the isolates of *Pseudostaurosira* were similar). PCA analysis demonstrated differences within raphid (*Achnanthes* and *Cymbella*) and araphid (all chain forming diatoms in this study) diatoms.

Both *Cymbella* species and *A. minutissima* showed an overall distinct monosaccharide profile. However, *Pseudostaurosira*, *Staurosira* and *Punctastriata* were grouped together which were previously classified in the *Fragilaria* genus. Rearrangement of genus *Fragilaria* was based on morphology and separated it in to 5 or more genera. *F. construens* and *F. brevistriata* are now under genus *Staurosira* and *Pseudostaurosira* respectively (Williams, 2006; Williams & Round, 1987). In our study, phylogenetic analysis based on 18S rDNA sequences homology demonstrated that the isolates of *Pseudostaurosira*, *Staurosira* and *Punctastriata* form a heterogeneous group (Chapter 4). Similarly, PCA also revealed that the monosaccharide signature of these genera form a group, although, it is as heterogeneous as in the phylogenetic tree.

In conclusion,

1. We optimized a protocol for successfully extracting EPS sequentially from araphid and raphid diatom species. In our isolates, cell-cell attachment is mediated by intercellular pads (araphid) hot bicarbonate treatment could successfully extract the EPS from such intercellular pads thus breaking the long chains to shorter.
2. Significant differences were observed in the monosaccharide composition between all the EPS fractions of a single isolate, whereas phylogenetically

related isolates of single species when compared for monosaccharide composition in individual components showed similarities in most of the cases.

3. Based on PCA it was shown that most of the araphid diatoms grouped together. *F. capucina* is phylogenetically distinct from *Pseudostauroopsis*, *Staurosira* and *Punctastriata*. In PCA, it was also separated from the other araphid diatoms. Profiles of raphids were well separated from araphids.

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## Chapter 8

### Diatom associated bacteria and consumption of diatom derived EPS: a study from epilithic biofilms in Lake Constance

Rahul A. Bahulikar, Monali C. Rahalkar, Christian Bruckner, Bernhard Schink and  
Peter Kroth\*

Faculty of Biology, University of Konstanz, University str. 10, Konstanz Germany

**First three authors contributed equally**

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Abbreviations: EPS, extracellular polymeric substances; soluble EPS, SE;

**Abstract**

The presence of bacteria was observed in uni-algal non-axenic diatom cultures isolated from freshwater epilithic biofilms of Lake Constance. The composition of diatom associated bacterial communities was studied in 14 different diatom cultures. There was a clear dominance of  $\alpha$ -proteobacteria observed in almost all diatom cultures, followed by  $\beta$ -proteobacteria,  $\gamma$ -proteobacteria, CFB and *Verrucomicrobia*. Furthermore, we tried to isolate bacteria from natural biofilms which could grow in diatom spent medium. We were able to isolate strains, which were previously uncultured, thus presenting an attractive approach for isolating novel bacteria.

## Introduction

In aquatic systems, the major interaction of bacteria with phytoplankton is the degradation and cycling of organic matter produced by the phytoplankton. (Grossart et al., 2005; Schäfer et al., 2002). The role of specific classes of bacteria involved in such degradation of particulate organic matter (POM) and dissolved organic matter (DOM) released by diatoms and other algae has been studied in marine (Riemann et al., 2000) as well as in freshwater systems (Makk et al., 2003; Riemann & Winding, 2001). Lake Constance is a warm, monomictic, prealpine lake and the bacterial communities active in different micro and macro-aggregates including lake snow have been well studied (Brachvogel et al., 2001; Grossart et al., 1997; Schweitzer et al., 2001).

Our study focuses on another of such miniature ecosystems: epilithic (growing on stones) biofilms, in the shoreline of littoral zone of Lake Constance. Epilithic biofilms are examples of complex communities where interactions are going on between the primary producers which are mainly the diatoms, algae and bacteria, fungi, protozoa, insects etc. (R. Bahulikar, unpublished data; Makk et al., 2003). Epilithic biofilms or periphyton from Lake Constance has been the subject of our recent work where we studied the dynamics of diatom communities in these biofilms on a spatial (Chapter 2) and temporal scale (Chapter 3). The use of taxa-specific quantitative PCR (Fierer et al., 2005) indicated the dominance of  $\beta$ -proteobacteria, High GC gram positives- *Actinobacteria* and *Cytophaga-Flavobacterium-Bacteroides* (CFB) related bacteria in these epilithic biofilms (Chapter 2).

Algal cultures are usually isolated as single cells by micro-manipulation techniques, and such non-axenic uni-algal cultures harbor a distinct assemblage of associated bacteria, which also have been termed satellite bacteria (Bell, 1984; Schäfer et al., 2002). Usually such algal cultures are maintained for several years and thus the associated bacteria also undergo selection. Such satellite bacteria associated with marine diatoms have been studied previously and were found to be mainly members of  $\alpha$ -proteobacteria or the CFB group (Schäfer et al., 2002). The CFB group has been mainly thought to be responsible for the breakdown of macromolecular organic matter and  $\alpha$ -proteobacteria for the utilization of carbon of small molecular mass (Cottrell & Kirchman, 2000). Communities of bacteria associated with freshwater diatoms from Lake Constance have not been studied yet.

As mentioned earlier, biofilms represent a complex system in which different members react with each other and it is difficult to study the interactions between each partners at the same time. As diatoms are the major primary producers and the early colonizers of surfaces (Ács, 1998; Nandakumar et al., 2004), studies of associated bacteria from such uni-algal cultures could also reflect their probable role in natural biofilms in degradation of complex organic matter produced by the diatoms. Diatoms are known to produce copious amounts of extracellular polymeric substance (EPS) which is an important source of carbon for heterotrophic bacteria (Giroldo et al., 2003). Thus there might be a phycosphere in the close vicinity of diatoms or algae, which release different organic compounds, which are utilized by these satellite or phycosphere bacteria (Schäfer et al., 2002). We found that usually diatoms isolated from epilithic biofilms had associated bacterial populations which were often difficult to remove, even with various combinations of antibiotics. Therefore we investigated these bacterial populations, by molecular and culture based approaches. In our study, we report the occurrence and diversity of bacteria which are associated with uni-algal diatom cultures that were isolated from epilithic biofilms of Lake Constance.

The main aims of this study were - 1. To study the community structure of diatom associated bacteria in uni-algal diatom cultures isolated from epilithic biofilms of Lake Constance. 2. To study the similarities and differences in the associated communities. 3. To investigate the role of diatom exudates in the spent medium, as a natural substrate for bacteria.

## Materials and methods

### *Diatom cultures*

For isolation and cultivation of diatoms, 5-10  $\mu$ l of biofilm was scraped from stones collected from littoral zone of Lake Constance, diluted by 1.0 - 1.5 ml of sterile DM (diatom medium) (Watanabe, 2005) and mixed many times with a pipette. Single diatom cells were isolated by micro-manipulation technique and cultured on DM (Table 1). These cultures were grown under 16 h at 16 °C 50  $\mu$ E illumination was provided by cool white fluorescent tube lights. The cultures were subcultured monthly and maintained in the same medium. Fourteen uni-algal diatoms cultures were used for this study (Table 1)

Table 1: Isolate number and names of the diatom species used for this study. These numbers are prefixed with the bacterial clones in the phylogenetic trees

Isolate number	Name of the diatom
D-02	<i>Stephanodiscus</i> sp.
D-12	<i>Pinnularia viridis</i>
D-16	<i>Synedra angustissima</i>
D-20	<i>Staurosira</i>
D-55	<i>Cymbella lanceolata</i>
D-92	<i>Navicula radiosa</i>
D-124	<i>Gomphonema truncatum</i>
D-04	<i>Amphora ovalis</i>
D-23	<i>Cymbella microcephala</i>
D-44	<i>Asterionella ralfsii</i>
D-45	<i>Diatoma tenuis</i>
D-14	<i>Cymatopleura solea</i>
D-48	<i>Pseudostauroopsis</i> sp.
D-53	<i>Cymbella subturgidata</i>
I-51	<i>Cymbella minuta</i>
I-04	<i>Cymbella microcephala</i>
B-04	<i>Cymbella microcephala</i>

### *DNA extraction and 16S rRNA gene clone libraries*

Diatom cultures, which were subcultured at least 4-5 times, were used for this study. Cultures were harvested at stationary phase, centrifuged and the cell pellet (Table 1) was frozen under liquid nitrogen and crushed using small pestle. 1 ml of prewarmed CTAB extraction buffer (Murray & Thompson, 1980) was added to it and incubated at 65 °C for 1 h. Then it was cooled to room temperature and chloroform : IAA (24:1) wash was given. DNA was precipitated using 0.7 volumes isopropanol which was followed by 80% ethanol wash and drying. The dried DNA was dissolved in 100 µl 10 mM : Tris 1 mM EDTA (TE) buffer. 50 ng of total DNA was used to amplify 16S rRNA genes using the universal bacterial primers 27 f (Edwards et al., 1989) and 1492 r (Weisburg et al., 1991). For amplification of the 16S rDNA genes, an initial denaturation at 94°C for 3 min was done, followed by 32 cycles at 94 °C for 30 sec, 53 °C for 30 sec and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. Amplified products were checked on 1% agarose gel electrophoresis and purified using NucleoSpin kit (Machery-Nagel, Germany) followed by cloning using the pGEM-T cloning kit (Promega, Germany). Around forty to fifty randomly selected colonies were subjected to tooth-pick PCR using enzyme *Msp* I. Restriction patterns were analyzed using 2% Nu-Sieve agarose (NuSieve ® 3:1 Agarose, Cambrex Bio Science Rockland Inc., ME) gel electrophoresis. These restriction patterns were compared with patterns from other clone libraries and common patterns were found.

### *Sequencing and Phylogenetic analysis*

At least 10% plasmids of each unique pattern were sequenced. The sequence reactions were prepared with either the dye primer cycle sequencing ready reaction (SP6 and T7 primers) kit or the PRISM ready reaction dideoxy termination cycle sequencing kit (Perkin-Elmer). Sequences were then obtained using an Applied Biosystems (model 3700) automated sequencer. Blast search was performed at the NCBI site (<http://www.ncbi.nlm.nih.gov/>) (Altschul et al., 1990) and closely related sequences were retrieved. All sequences were checked for chimeras by dividing the sequence in two partial sequences and performing blast search and phylogenetically analysed using the ARB software package (version 2.5b; <http://www.arb-home.de>) (Ludwig et al., 2004). The new sequences were added to the ARB database and aligned using the FAST Aligner tool as implemented in ARB. Alignments were checked and manually corrected where necessary. Sequences with more than 1400

nucleotides were used for alignment. Specific filters were used for specific phylogenetic classes available in the ARB database. Phylogenetic analysis was done using the maximum likelihood, neighbour-joining and maximum parsimony algorithms as implemented in ARB.

*Isolation of axenic diatoms and their growth*

Unialgal axenic diatoms were isolated as described (Bahulikar & Kroth in preparation). Four such axenic diatom cultures from the genus *Cymbella* were used. *C. microcephala* (isolates B-04, I-04 and D-23) were grown in 1 l flasks containing 600 ml DM medium (Watanabe, 2005) and were allowed to grow till stationary phase, whereas, *C. minuta* (isolate I-51) was grown in continuous culture. Diatom cells were removed by centrifugation at 10,000 rpm for 10 min and the spent medium was used for the further experiment. The spent medium was microscopically observed for the remnants of diatom cells or any contaminating bacteria. Carbohydrate assay (Dubois et al., 1956) was used to measure soluble EPS before and after bacterial growth.

*Utilization of diatom derived soluble EPS by bacteria*

Stones from the littoral zone of Lake Constance from 20-30 cm water depth were collected on 19<sup>th</sup> August 2006. The epilithic biofilms were scraped off and pooled in a Falcon tube. Around 2.5 ml of the biofilm material was scraped and diluted 10 times with DM to 25 ml. The tube was vortexed vigorously for two to three minutes, until most of the particles were well suspended, mixed well by pipetting and then was serially diluted 1: 10 upto  $10^{-8}$ . At each step the tubes were vortexed for 1 - 2 minutes for dispersing the bacteria evenly. 5 ml from each of these dilutions were used for inoculating 45 ml of the spent medium from diatom cultures containing soluble EPS in 100 ml flasks. Un-inoculated flasks served as controls and were also incubated. These flasks were incubated at 26 -27 °C in dark for 15 days without shaking. The contents were mixed after 3-4 days and then again incubated. The final OD was recorded on the 15<sup>th</sup> day and samples were taken on day 8 and day 15 for the estimation of soluble EPS. The soluble EPS was measured as described before.

*Isolation and characterization of bacteria*

Two last positive dilutions were used for isolating the dominant bacteria utilizing the diatom produced EPS in the spent media. Bacteria from lower dilutions were also plated out to see the overall diversity. Around 20 µl of these dilutions were streaked on to two different agar plates and incubated at 23 °C till colonies were observed. The two different solid media were 1:2 diluted nutrient agar with 10 mM

glucose and 1:10 dilute nutrient agar with 10 mM glucose with 1.5 % agar (DIFCO). After visible colonies appeared, the bacteria were observed under a phase contrast microscope (Axiophot; Zeiss, Oberkochen, Germany), restreaked and purified, until pure or nearly pure cultures were obtained. A total of 24 colonies were analyzed further. A colony was suspended in few  $\mu$ l of 10 mM Tris, 1 mM EDTA buffer, boiled at 90 °C for 10 min. and the 16S rDNA was amplified followed by RFLP analysis as described before. Unique representative strains (a total of 3) were sequenced either partially or fully and the sequences were added to the phylogenetic trees created before.

## Results

### *Diversity of the associated bacteria*

A total of fourteen diatom strains were used for the investigation of the associated bacteria. Even though these diatoms represent different genera, the bacteria associated with each diatom culture showed a common pattern. From a total of 520 clones analyzed by RFLP, diatom plastids contributed to a large portion of the clones (59%), although bacteria were present in all diatom cultures. Within the bacterial community a clear dominance of  $\alpha$ -proteobacteria was seen (59.19% of total bacterial clones).  $\beta$ - and  $\gamma$ -proteobacteria contributed for 13% each, followed by the CFB group (11%) whereas, some of the clones (3%) were related to *Verrucomicrobium*. After phylogenetic analysis it was found that most of the sequences from the present study formed close clusters or distinct groups. Within  $\alpha$ -proteobacteria, a total of five different clusters were found which mainly consisted of clones obtained in this study (Fig.1). Clones from  $\alpha$  Cluster 1 were related to *Erythromicrobium* – *Porphyrobacter* group. Two clusters belong to sphingomonads ( $\alpha$  Cluster 2a and b). Some clones were related to *Rhodobacter* ( $\alpha$  cluster 3).  $\alpha$  Cluster 4 and 5 were related to *Brevundimonas* and *Azospirillum*, respectively.  $\beta$ -proteobacteria were mainly related to *Acidovorax* sp. or *Aquamonas* (Fig. 2). Most of the  $\gamma$ -proteobacteria were *Pseudomonas* sp. In the CFB group, bacteria were related to *Flavobacterium* or *Sphingobacterium* genera (Fig. 3).

### *Utilization of diatom EPS by bacteria from epilithic biofilms*

Four axenic diatom strain belonging to the *Cymbella* genus were used for studying the bacterial utilization of diatom derived EPS. These diatoms have been isolated from Lake Constance and are known to produce high amounts of EPS (Chapters 6 and 7) in addition to being dominant diatoms from epilithic biofilms of Lake Constance (Chapters 2 and 3). All *C. microcephala* species showed higher amount of galactose (40-42%) and mannose/xylose (32-43%) in the EPS and EPS from *C. minuta* mainly contained galactose (52%) and mannose/xylose (31%) (Chapter 6).

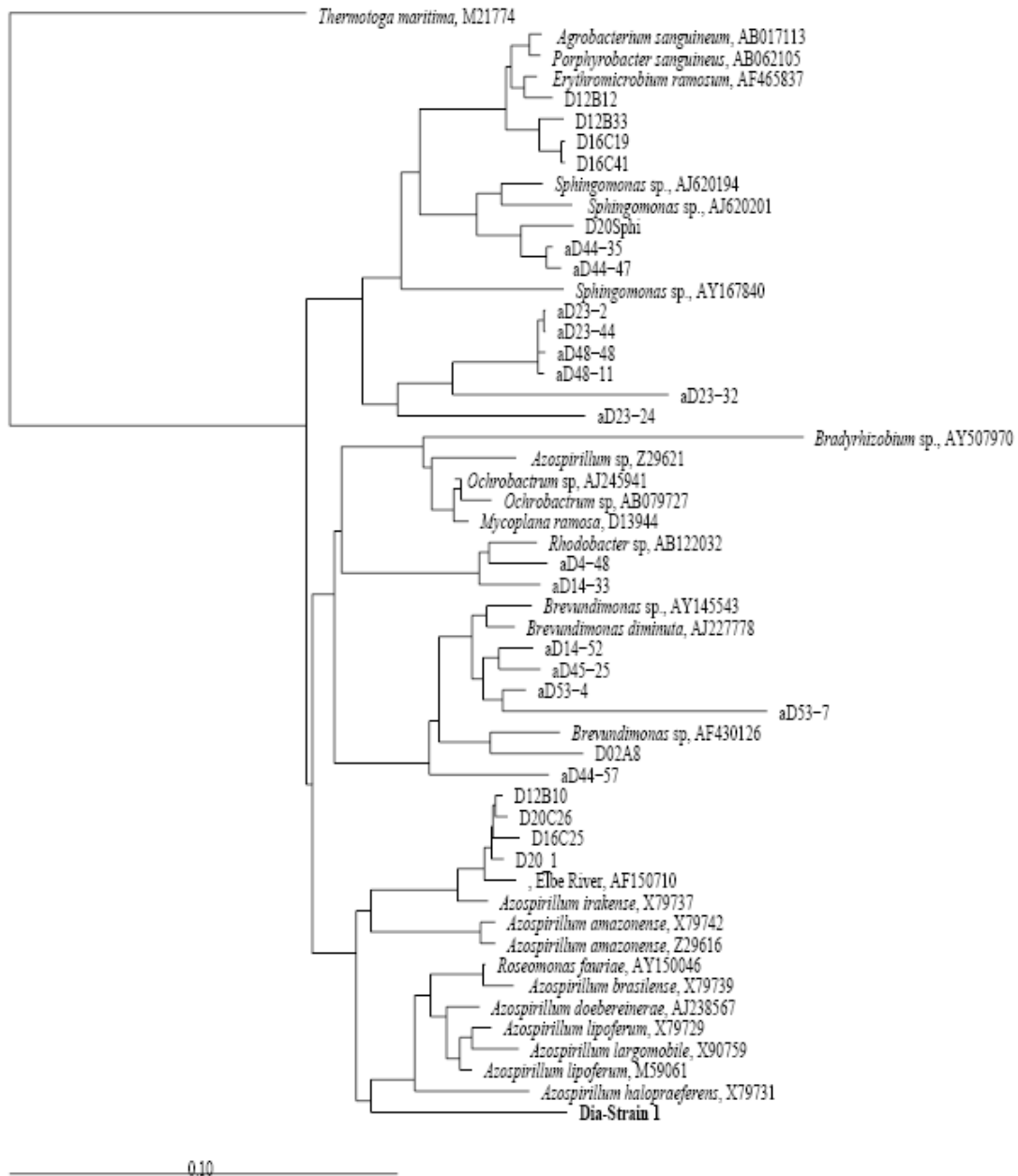


Fig. 1 Rooted phylogenetic tree calculated by neighbor joining method showing 16S rDNA sequences recovered from the clone libraries of diatom associated bacteria from the  $\alpha$ -proteobacteria. Clones obtained from our study are denoted as D## or aD##- followed by the clone number. Representatives of cultured and uncultured  $\alpha$ -proteobacteria were used for the analysis and nearly complete sequences >1400 nucleotides were considered. NCBI accession numbers of clones and cultures are given and bar represents 10% divergence. The tree was rooted with *Thermotoga maritima* as the out-group.

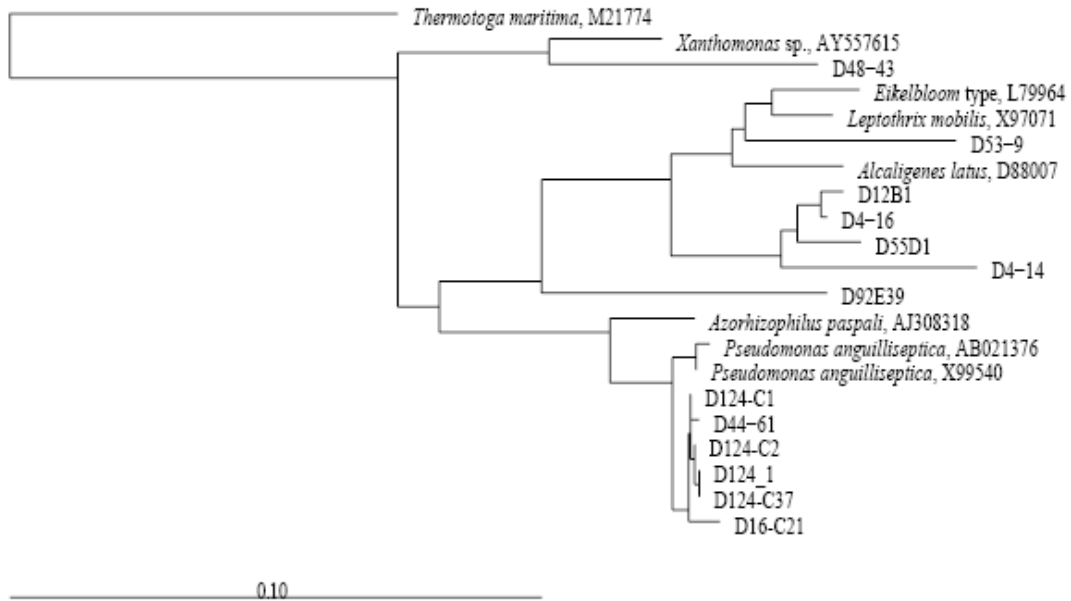


Fig.2 Rooted phylogenetic tree calculated by neighbor joining method showing 16S rDNA sequences recovered from the clone libraries of diatom associated bacteria from the  $\beta$ - and  $\gamma$ -proteobacteria. Clones obtained from our study are denoted as D## followed by the clone number. Representatives of cultured and uncultured  $\beta$ - and  $\gamma$ -proteobacteria were used for the analysis and nearly complete sequences >1400 nucleotides were considered. NCBI accession numbers of clones and cultures are given and bar represents 10% divergence. The tree was rooted with *Thermotoga maritima* as the out-group.

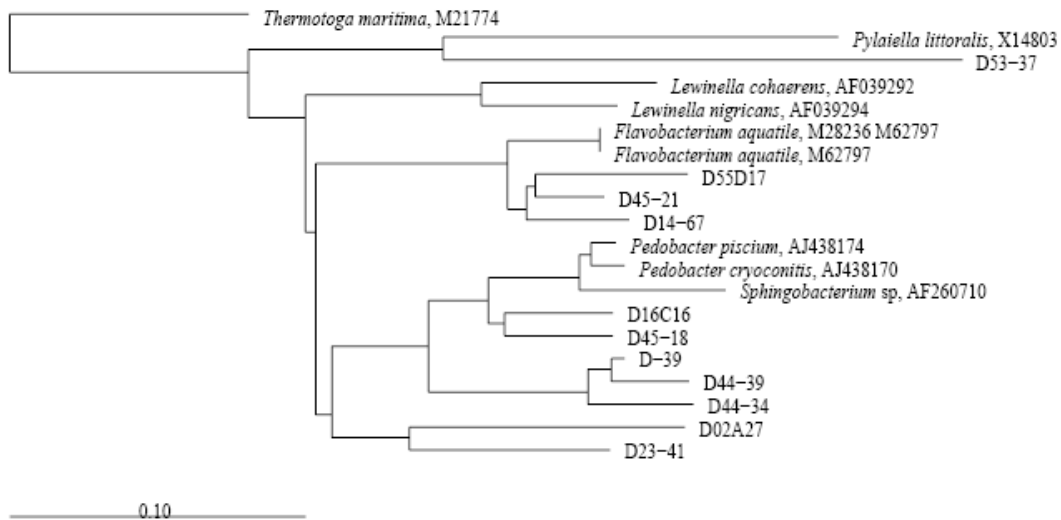


Fig. 3 Rooted phylogenetic tree calculated by neighbor joining method showing 16S rDNA sequences recovered from the clone libraries of diatom associated bacteria from the *Cytophaga/Flavobacteria/Bacteriodes* (CFB). Clones obtained from our study are denoted as D## followed by the clone number. Representatives of cultured and uncultured CFB were used for the analysis and nearly complete sequences >1400 nucleotides were considered. NCBI accession numbers of clones and cultures are given and bar represents 10% divergence. The tree was rooted with *Thermotoga maritima* as the out-group.

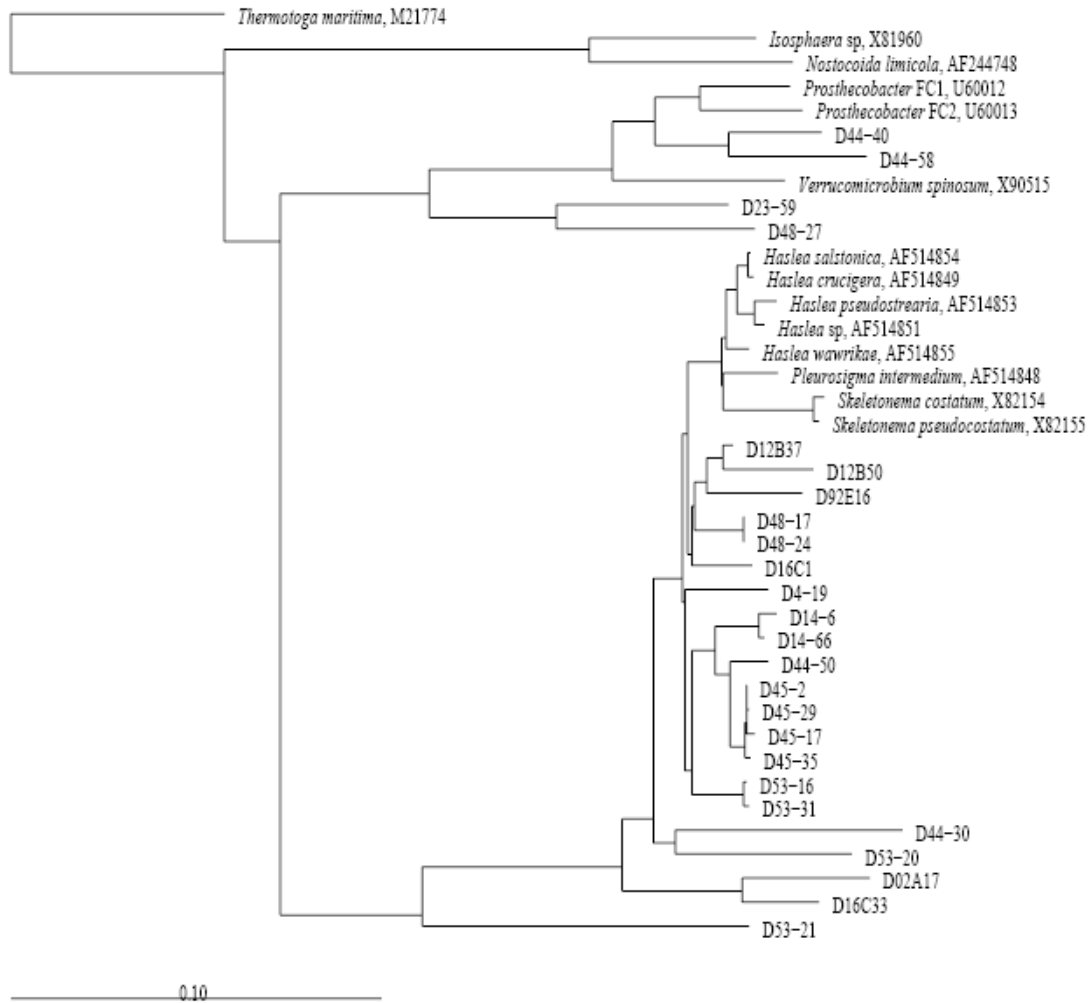


Fig. 4 Rooted phylogenetic tree calculated by neighbor joining method showing 16S rDNA sequences recovered from the clone libraries of diatom associated bacteria from the *Verrucomicrobium* and 16S rDNA sequences from chloroplasts of diatom isolates. Clones obtained from our study are denoted as D## followed by the clone number. Representatives of cultured and uncultured *Verrucomicrobium* and 16S rDNA sequences from chloroplasts of diatom isolates were used for the analysis and nearly complete sequences >1400 nucleotides were considered. NCBI accession numbers of clones and cultures are given and bar represents 10% divergence. The tree was rooted with *Thermotoga maritima* as the out-group.

#### *Growth experiments using diatom spent medium*

As sugars (soluble EPS) are the major component in the spent medium of diatoms (Hoagland et al., 1993; Underwood & Paterson, 2003) the soluble sugar utilization by bacterial community was monitored. The flasks containing spent medium were inoculated with epilithic bacterial community and were sampled after 8 days and 15 days (Fig. 5). The initial amount of soluble EPS was different in all of the diatom spent media used. The rate at which this EPS was used was different with each diatom isolate. In the spent medium of *C. microcephala* (I-04) the initial EPS

provided was high i.e. around 80-90  $\mu\text{g/ml}$ . Within 8 days, in the  $10^{-6}$  dilution around half of the sugar was utilized, whereas within 15 days most of it was utilized (Fig. 5A). In  $10^{-7}$  dilution, growth was observed although the EPS was not completely utilized. In case of the spent medium of the second isolate of *C. microcephala* (B-4), large amount of EPS was already utilized within the first 8 days and growth was observed till the last dilution step i.e.  $10^{-9}$  final dilution. After 15 days most of the EPS was utilized and there was almost no EPS left in the lower dilutions i.e. upto  $10^{-7}$  (Fig. 5B). Third isolate of *C. microcephala* secreted low amounts of EPS ( $\sim 20 \mu\text{g/ml}$ ) most of which was utilized within 8 days (Fig. 5C). Spent medium of the isolate I-51 belonging to *Cymbella minuta* had very high amounts ( $\sim 110 \mu\text{g/ml}$ ) of EPS in the spent medium which was not utilized within the first 8 days but then after 15 days a major part was consumed (Fig 5D).

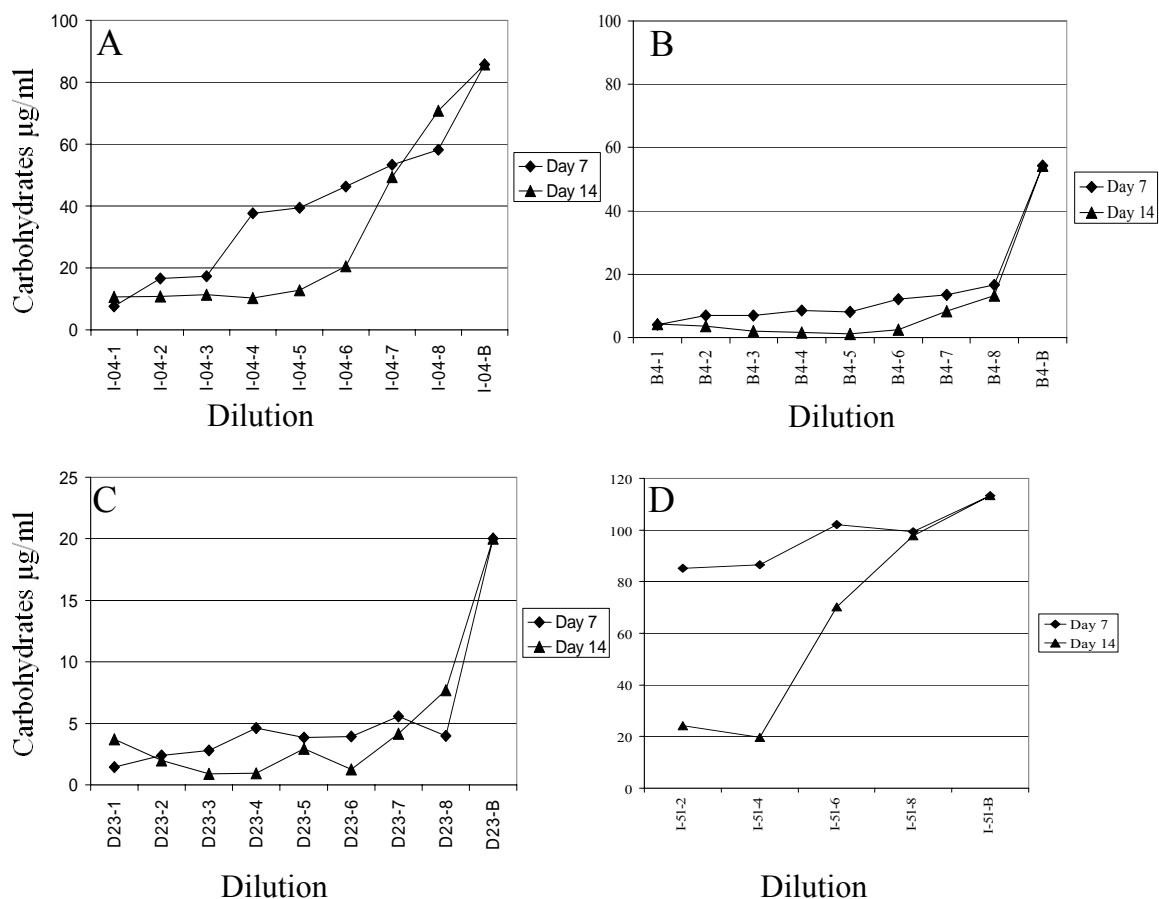


Fig. 5 Consumption of sugars from spent media from various diatom isolates by a serial dilution series derived from the epilithic biofilm as an inoculum. X-axis shows the dilution step (flask) and Y axis shows the concentration of the sugar measured in a particular dilution step. These measurements were done after 7 days and 15 days after inoculation.

Bacteria growing in the highest dilutions would represent the most dominant bacteria which might be involved in degradation of the diatom exudates. In all 4 cases growth was observed up to  $10^{-8}$  dilution. Bacteria were successfully isolated in media from the  $10^{-8}$  dilutions in most of the cases.

*Novel Bacteria isolated from the enrichments*

Various types of colonies were observed in the lower dilutions plated, whereas only few colony types were observed in the last positive dilutions. Colonies isolated from the last positive dilutions of I-04 and B4, were milky white or yellowish in color. Five isolates obtained from the I-04 spent medium showed a similar RFLP pattern and were morphologically similar i.e. spiral motile rods. Partial sequencing of 16S rDNA was done with one of the representative strains, named as dia-strain 1, belonged to the Rhodospirillaceae family, and was 90-91% similar to *Azospirillum* sp. (closest relative *A. lipoferum*). Phylogenetic analysis of this strain also placed it close to the *Azospirillum* group and further characters of this strain are being investigated.

The other five to six strains were isolated from B-4, D-23 and I-51 partially sequenced (16S rDNA) and were found to belong to the *Pseudomonas* genus. Two colonies were morphologically similar to *Micrococcus* (isolated from B4) and one to *Erythromicrobium* (isolated from I-51).

## Discussion

Diatoms are one of the early colonizers of surfaces such as stones in freshwater systems like lakes, rivers and streams (Ács et al., 2000; Peterson & Hoagland, 1990; Sekar et al., 2002; Sekar et al., 2004). Along with bacteria, they form biofilms on these surfaces and such epilithic biofilms are often dominated by diatoms (Ács et al., 2003; Sherwood & Sheath, 1999). Our study mainly focuses on the fact that bacteria and diatoms do coexist in nature as well as in artificial culture systems (Grossart et al., 2005; Hoagland et al., 1993; Schäfer et al., 2002). We wanted to know whether diatoms and bacteria do have any specific community composition. A further question was, 'whether the photosynthetic partner can support the growth of its heterotrophic counter part.' The reason for co-occurrence of such satellite bacteria in marine diatom cultures has been addressed in detail before (Bell, 1984; Schäfer et al., 2002). Bell (1984) has suggested that the low molecular mass compounds secreted by algae are mainly utilized by bacteria. Polysaccharides are known to be the major part of soluble EPS produced by the diatoms. They are water soluble and secreted into the medium. Thus, we used spent medium obtained from diatoms to analyze growth of epilithic bacteria.

### *Diversity of associated bacteria*

In our study,  $\alpha$ -proteobacteria dominated the clone libraries of diatom associated bacterial communities. Although, the results presented with respect to the abundances of the different classes of bacteria are cumulative, the individual clone libraries of diatom-associated bacteria were also mainly dominated mainly by  $\alpha$ -proteobacteria (details not shown). Bacteria associated with lake snow aggregates derived from phytoplankton, mainly diatom blooms have been well studied by clone library approach as followed by FISH (Fluorescence in situ hybridization) with the specific dominant groups within each phylogenetic group (Schweitzer et al., 2001). A clear dominance of  $\alpha$ - and  $\beta$ -proteobacteria was observed followed by the CFB group in later stages of the diatom bloom. Within  $\alpha$ -proteobacteria, there was a dominance of a group of clones related to *Sphingomonas* – *Caulobacter* – *Rhizomonas* and another group of clones related to *Brevundimonas* – *Mycoplasma*. By applying FISH probes specific for *Sphingomonas* spp. and *Brevundimonas dimunita*, 16-60% of  $\alpha$ -proteobacteria were detected in lake snow microaggregates (Simon et al., 2002). In our study, we found both of these clusters (i.e. *Sphingomonas* and *Brevundimonas*),

and additionally clones related to other  $\alpha$ -proteobacterial subgroups like *Erythromicrobium-Porphyrrobacter*, *Azospirillum* and *Rhodobacter*. Bacteria assemblages associated with certain marine diatom species have also been shown to be dominated by  $\alpha$ -proteobacteria mainly of the *Roseobacter* clade (Allgaier et al., 2003). *Sphingomonas* related bacteria are well known to degrade various pollutants (Schweitzer et al., 2001). Interestingly, *Azospirillum* related-clones were frequent. *Azospirillum* spp are known to be nitrogen fixers and associated with roots of grasses and cereal crops (Peng et al., 2006). Further studies on specific interaction of *Azospirillum*-like bacteria with diatom cultures or in natural diatom assemblages would be very interesting.

Two marine diatoms, *Thalassiosira* and *Skeletonema*, Grossart et al. (2005) showed that the  $\alpha$ -proteobacteria and the bacteria of the CFB group dominate the free living and attached community, respectively. Similar observations have been done in a study of bacterial communities during a diatom bloom (Riemann et al., 2000).

Though we did not separate these 2 communities i.e. free living and attached bacteria while preparing the clone libraries, we found both of these groups, to be important members of the diatom associated bacterial community. The CFB group has been shown to be one of the dominant groups of bacteria in epilithic biofilms from Lake Constance, at the same study site at water levels of 20-50 cm, (Chapter 2). This group is mainly known to be involved in the degradation of larger molecules and complex polymers (Kirchman, 2002).

$\beta$ -proteobacteria have also been shown to be one of the dominant groups in diatom derived microaggregates (Brachvogel et al., 2001), in lake snow of Lake Constance (Schweitzer et al., 2001) represented mainly by the *Hydrogenphaga*, *Acidovorax* group. In this study, we found that  $\beta$ -proteobacteria were related exactly to the same groups, in addition to *Aquaspirillum* and *Aquamonas*. Recently, our study on the abundances of different classes of bacteria in epilithic biofilms of Lake Constance has revealed that  $\beta$ -proteobacteria dominated in older biofilms, i.e. occurring in deeper water, after the spring water level increase. Their abundance correlated inversely with the soluble EPS found in these biofilms, indicating a possible role in its consumption (Chapter 2). *Pseudomonas* species are ubiquitous and are capable of degrading a variety of substrates (Ács et al., 2003). In our study, *Pseudomonas anguilliseptica* related bacteria dominated the  $\gamma$ -proteobacterial clone libraries. *P. anguilliseptica* is known to be a fish pathogen (Doménech et al., 1999). A

minor amount of clones also belonged to *Verrucomicrobia* and *Planctomycetes*, the later have been found to be a dominant group in river biofilms (Brümmer et al., 2004).

*Diatom spent medium: a dilute natural substrate*

It is well known that culturability of some bacteria in the laboratory is very low (in some cases as low as 0.001% of total) (Gich et al., 2005). One third of the bacterial divisions consist of un-cultured bacteria, and many well-known divisions such as  $\beta$ -proteobacteria in fresh water lakes contain novel uncultured lineages. (Bruns et al., 2003). Thus there is always a need to improve the existing methodology and to have novel cultivation dependant approaches to understand the functional role of bacteria in the environment (Bruns et al., 2003; Watve et al., 2000).

Most of the bacteria in the environment are exposed to low substrate concentrations and high substrates supplied to them in the laboratory media could cause substrate accelerated death (Bruns et al., 2003; Watve et al., 2000). Even in metabolic hotspots like biofilms the concentration of soluble sugars were low, in the range of 20-800 mg/l (i.e. 0.002-0.08 w/v), compared to the routine concentrations used in standard bacteriological media. Use of dilute media has thus been proven to be important in increasing the efficiency of bacterial culturability (Bruns et al., 2003; Watve et al., 2000).

In the present study, we found that since bacteria co-exist with diatoms, the compounds secreted by diatoms could be useful for the growth of bacteria. Although, it would be very interesting to explore if the other way round, i.e. do bacterial interaction with the diatoms (Croft et al., 2005). We explored the possibility of providing diatom spent medium as a nutrient source for cultivation of bacteria from a natural source i.e. epilithic biofilms. EPS by secreted diatoms contains mainly polysaccharides (Wustman et al., 1997), low amounts of proteins (Daniel et al., 1987)/ glycoproteins (Chiovitti et al., 2003) and uronic acids (Abdullahi et al., 2006; Chiovitti et al., 2003). Thus the diatom spent medium could provide a variety of substrates in low concentrations. Since carbohydrates are the main components, we could measure then and follow the dynamics of their utilization. It was seen that two species of the same diatom genera produced different amounts of soluble sugars. In our study, we found that the spent medium allowed cultivation of bacteria from biofilm up to  $10^8$  -  $10^9$  cell/ ml, and showed that such natural substrates can be used for cultivation of bacteria.

*Isolation of previously uncultured bacteria*

The spent medium from diatoms allowed us to cultivate a large number of bacteria and it also enabled us to isolate and cultivate bacteria which are phylogenetically new and could represent a new taxon. The presence of *Azospirillum*-like bacteria in the diatom-associated bacterial cultures was indicated in the clone libraries of diatom-associated bacteria. Further we could directly isolate a novel relative of *Azospirillum*-like bacteria, from the Rhodospirillaceae family. As Dia-strain 1, is only 91% related to *Azospirillum*, it could represent a new bacterial taxon, after phylogenetic and physiological characterization. Since *Azospirillum* is a nitrogen fixer and associated with grasses, the nitrogen fixation abilities of Dia-strain 1 could be also explored.

**Acknowledgements**

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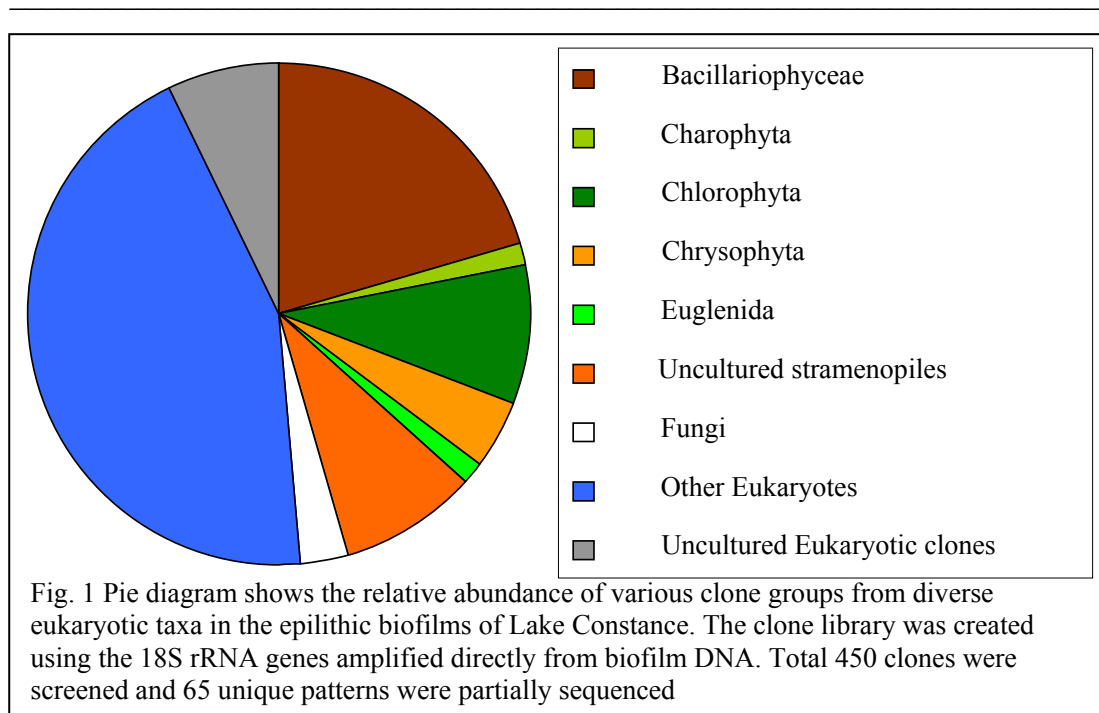
## Chapter 9

### General discussion

### **Diatom diversity**

Margin of lake is generally composed of stones or gravel, whereas, in deeper areas it consists mainly of fine sediment (Stevenson et al., 1996). The margin of the lake is affected by high-energy waves, water level fluctuations and high temperature differences (Stevenson et al., 1996). On the gravel as well as on the stones a thin layer of biofilm is present and is colonized by various species of algae, bacteria, fungi, insects, nematodes. These biofilms are ecologically important, as they are metabolic hotspots. Primary producers fix carbon photosynthetically and fuel a complex food web (Smetacek, 1999). Diatoms are the major primary producers in benthic and pelagic zones. CO<sub>2</sub> fixed in the benthic zone is comparable with the pelagic zone (Wetzel, 1964). Diatom communities from pelagic zone of Lake Constance have been studied in great details in the last decade (Sommer, 1984; Sommer, 1985; Sommer et al., 1993; Sommer & Stabel, 1983), whereas no reports available on benthic diatom communities from the epilithic biofilms of littoral zone of Lake Constance. Efforts have been taken to document the diatom community structure, their EPS composition and interactions with bacterial communities in epilithic biofilms.

First, a molecular approach was used to study the eukaryotic diversity of an epilithic biofilm. An 18S rDNA clone library was constructed using DNA extracted from biofilm and was screened by RFLP. Clones with unique patterns were sequenced partially and compared with sequences in the GenBank. This analysis yielded information about the overall eukaryotic diversity, which is present in the biofilm. We detected the presence of various algae fungi, nematodes and insects. Diatoms were the dominant algae among various others with respect to species diversity (Fig. 1). Dominance of diatoms over other algal groups in terms of cell number and species richness was also recorded in various benthic environments such as lakes (Ács et al., 2003), streams (Soininen, 2004; Soininen & Könönen, 2004), rivers (Ács, 1998; Ács & Kiss, 1993; Barreto et al., 1997; Soininen, 2002) and intertidal mudflats (Mitbavkar & Anil, 2002; Underwood, 1994). Diatoms are known to play an important role in pioneering new surfaces and for establishment of biofilms (Ács, 1998; Tuchman & Stevenson, 1991).



The diatom community structure was analyzed at five nearby locations (50 m) and at four different depths (Chapter 2). We also studied seasonal fluctuations in the diatom communities where biofilms were collected throughout the year (Chapter 3). For the former study, samples were collected during increasing water level during (April 2005). Areas of sample collections were dry during January 2005 and as water level rises, the biofilms formation took place on the reflooded stones. We used the increasing water levels as a natural time scale and studied the trends in two important components of epilithic biofilms, i.e. diatom and bacteria, across a depth gradient. Interestingly, species richness was higher in 20 cm depth and showed a slight decrease at 30 and 40 cm, followed by higher species richness again in deeper areas. This trend was observed in almost all locations. When compared with water level data, biofilms at lower depths were still developing and facing high disturbances due to waves compared to deeper sites. The biofilms in 50 cm depth appeared relatively thicker and mature as compared to the biofilms at lower depths. Principal component analysis of the diatom communities revealed that samples from the same depth from different locations grouped together, which implies that water level was an important factor influencing the community structure. The deeper biofilms are generally not affected by high energy waves (Peterson et al., 1990), which is one of the cause of degradation / disturbances in the biofilms. Grazing is also an important parameter for causing disturbances in the biofilm as selective grazing for instance might change the community structure (Tuchman & Stevenson, 1991). The same biofilm samples were

further analyzed for EPS content and for bacterial abundance by qPCR. The amount of chlorophyll *a* and soluble EPS content decreased according to depth, indicating that in young biofilms the primary production was the most important process, lasting to a higher content of soluble EPS. However, it was observed that the abundance of  $\beta$ -proteobacteria, CFB and HGC – *Actinobacteria* together increased with depths, indicating that these bacterial communities were getting more and more established in mature biofilms at deeper areas, which are known to be specialised in degradation of organic matter and are known to dominate diatom microaggregates (Brachvogel et al., 2001).

To get an overall idea about the diatom community structure in the epilithic biofilms it is necessary to observe the temporal or seasonal fluctuations, this was the objective of our further study (Chapter 3). Samples were collected from June 2004 – June 2005 and were from biofilms at water depths of 20-30 cm. Clear changes in the diatom community structure as well as EPS content in the pore water were observed. These changes might be due to different factors associated with changing seasons, mainly changes in temperature (Klarer & Hickman, 1975), water levels (Wei & Chow-Fraser, 2006) and light intensity (Hoagland & Peterson, 1990). Species richness was correlated with the changing seasons, e.g. *Denticula tenuis* and *Achnanthes minutissima* were abundant during summer and *Cymbella microcephala* was dominant during autumn, whereas a higher frustule count of *Amphora inariensis* was observed in winter.

In both studies, various diatom species were co-dominant i.e. they contributed to a significant percentage to the total community. These were *A. minutissima*, *Cymbella minuta*, *C. microcephala*, *D. tenuis*, *Diatoma vulgare* etc. Some of them were seasonally dominant while others represented a large portion of the total community throughout the year. Most of these species have been described as dominant members in the benthic biofilms (Stevenson et al., 1996). Amongst them *A. minutissima* and *C. microcephala* were dominant across various lakes in the epilithic biofilms (Barbiero, 2000). *D. vulgare* was found to be a pioneer species in biofilms on artificial substrata (Ács, 1998), whereas *A. minutissima* is known as a cosmopolitan species as it was reported in biofilms from various lakes (Ács et al., 2003; Ács & Buczkó, 1994; Hoagland & Peterson, 1990), rivers (Soininen, 2002; Soininen & Eloranta, 2004) and streams (Douglas, 1958; Sherwood & Sheath, 1999).

A common (Stal, 2003; Underwood & Smith, 1998) observation is a high correlation between chlorophyll *a* and EPS indicating that the EPS originates from photosynthesis, in such biofilms. Such a correlation was observed only when diatoms were the dominant members (Abdullahi et al., 2006; Stal, 2003). The EPS is used as a source of carbon by bacteria (Giroldo et al., 2003) or by diatom themselves in dark (de Brouwer & Stal, 2002; Tuchman et al., 2006).

Biofilm development or revival on the stones along shore-line usually occurs as the water levels increase in the spring. Supporting this hypothesis we observed high diatom diversity together with the increase of chlorophyll *a* and EPS during April 2005. Simultaneously increasing temperatures and or yearly water mixing also occur during this period. These factors are known to be important for the pelagic zone where the onset of thermal stratification resulted in algal blooms and biomass increases (Sommer, 1985). During this time many factors might be responsible for higher growth rates like increase in temperature, rising water levels and a lower number of grazers (Sommer, 1985).

### **Diatom cultivation and phylogeny**

For isolation and cultivation of diatoms from the natural biofilms, two main methods were used: micromanipulation techniques and spreading of diluted biofilms directly on agar media. Micromanipulation was found useful for isolation of specific and large sized diatom species. By manual selection, species that look different or conspicuous are picked up and minor differences in between different species cannot be judged. Whereas in the second method, the most abundant or dominant members can be cultivated. In addition, cultivation of small cell sized diatoms is possible, which is not achieved by micromanipulation. These techniques allowed us to cultivate more than hundred diatoms. In the isolates majority were from pennate diatoms, a few (3-4) were centric, and a similar distribution was observed in biofilms (Chapters 2 and 3).

Long-term maintenance of diatoms is often problematic. It is well known that the size of the diatoms goes on diminishing over time because mitotic cell division often results in daughter cells of smaller size. At critical cell size, diatoms have to undergo sexual reproduction to restore their maximum cell sizes (Round, 1982). However, sexual reproduction so far was only rarely observed in cultures and that could lead to the death of a culture. Therefore, the average age of most of the diatom

cultures was for a maximum of 2-3 years. Some diatom cultures e.g. *Cymatopleura* sp. and large sized *Cymbella* sp. were not viable in culture for more than a few months. Other species like *Gyrosigma*, in spite of several attempts of cultivation by the micromanipulation method, it failed to divide under culture conditions (personal observation). For unknown reason we could not cultivate the diatom *D. tenuis* which is likely abundant in the littoral zone of Lake Constance.

Diatom taxonomy has been based mainly on morphological characters but recently molecular analyses using 18S rDNA and various other genes have been proved to be useful to clearly elucidate the phylogenetic positions of controversial taxa (Kooistra et al., 2003; Medlin et al., 1988; Medlin et al., 1997). 18S rDNA sequence comparisons supported the monophyletic origin of diatoms within the heterokont lineage (Bhattacharya & Medlin, 1998; Medlin et al., 1993). Based on the morphological characters, diatoms are classified as centric and pennate diatoms. However, molecular analysis revealed a paraphyletic origin of centric diatoms (Medlin et al., 1996). Furthermore pennate diatoms are divided into two groups, raphids and araphids according to the presence and absence of the raphe and this character is supported by phylogeny and morphology of pennate diatoms (Medlin et al., 1993). The phylogenetic tree of raphid diatoms showed monophyletic origin (Medlin & Kaczmarska, 2004) but further absence of family wise clustering was observed (Chapter 4). Three of four families in this analysis were grouped in more than one cluster and a fourth formed a single group represented by very few diatom sequences (Chapter 4). Thus, diatoms of the raphid group showed paraphyletic nature. Molecular data from more diatoms and also various molecular marker based phylogenies would throw more light on this aspect.

Fragilaricaeae belong to the Araphid diatoms. On the morphological basis, the former genus *Fragilaria* has been divided into six genera (Williams & Round, 1987), whereas *Synedra* has been divided into twelve genera (Williams & Round, 1986). The systematics of *Synedra* and *Fragilaria* has been debated for long time (Williams, 2006). In our study, all the 18S rDNA sequences from various *Synedra* species grouped together and therefore might have a monophyletic origin. Even though the morphological characters are distinct amongst the *Fragilaria*-related diatoms, only *Nanofrustulum* was found to be monophyletic at the 18S rDNA level (Medlin, in preparation). This is in accordance with the revision based on Krammer & Lange-Bertalot (1986-1991) who combined all these taxa under a single genus, *Fragilaria*.

Further classification has not always been supported by molecular tools such as 18S rDNA sequence comparisons either in raphid pennates or in araphid pennates (Chapter 4 and Medlin in preparation). Even though there are more than 10,000 described species (Mann, 1999) not many sequences are available in the GenBank and also very few studies have been done on population genetics (Hwang & Tabita, 1991) (Créach et al., 2006; Wawrik et al., 2002). More work is needed on taxa specific studies as well as population genetics of diatoms, which will be useful to unravel phylogeography of particular taxa in a particular area.

### **Diatom Growth and EPS**

Diatoms are known to produce copious amount of EPS during their life cycle. Secretion of EPS by an axenic diatom species depends on two main factors such as nutrient availability and the growth phase. (de Brouwer & Stal, 2002; Staats et al., 1999). Diatoms produce two types of EPS, soluble EPS (SE) and bound EPS (BE). This secretion occurs as a result of an excess of fixed carbon and a metabolic overflow (Stal, 2003). SE is released in the surrounding water, whereas BE remains associated with the cell and may form secondary structures such as stalks, capsules, tubes or pads (Hoagland *et al.*, 1993).

Here we used two approaches to characterize BE: localization of specific structures formed using FITC labeled-lectins by fluorescence microscopy (Chapter 5 and 7) and chemical extraction, subsequent hydrolysis and HPLC analysis of monosaccharides.

In our study, higher amount of EPS production was specifically observed during stationary phase which has been also reported earlier (de Brouwer & Stal, 2002; Smith & Underwood, 2000). EPS production is also enhanced when nutrients are depleted (Abdullahi et al., 2006; Staats et al., 2000; Urbani et al., 2005). Among various diatoms, highest SE secretion was observed in all isolates of *C. microcephala*. The other species produced relatively lower amounts of the SE, which were comparable to those of various marine diatoms (de Brouwer & Stal, 2002; Staats *et al.*, 1999). Growth rates of isolates of *C. microcephala* were much higher than other diatoms. This diatom was dominant in the biofilm only during autumn, whereas *A. minutissima* was abundant throughout the year and was dominant during autumn in our study site. *A. minutissima* is also reported as a cosmopolitan species in various

water bodies (Ács *et al.*, 2003; Barbiero, 2000). The third diatom, *Pseudostauroopsis*, was also abundant throughout the year, but it was not dominant as *A. minutissima*.

Principal component analysis was used to correlate the monosaccharide profiles from the SE fractions from the diatoms *Cymbella microcephala*, *C. minuta*, *Achnanthes* and *Pseudostauroopsis* and the SE fractions from the pore water of two biofilms sampled showed that the SE profiles from both biofilms grouped with the profiles of *A. minutissima*. This indirectly suggests that *A. minutissima* might be responsible for contributing to the SE component in the epilithic biofilms from our study site in the littoral zone of Lake Constance. Although, its growth rate was lower in the culture condition, it is reported as small, fast growing, pioneer and dominant species (Ács *et al.*, 2003; Peterson *et al.*, 1990). It is also reported as a phosphate specialist species (Stevenson *et al.*, 1996). Success of *A. minutissima* in the epilithic biofilms might be due to lower concentrations of phosphate in Lake Constance (Bussmann *et al.*, 2004) or various other factors controlling biofilms such as high velocity waves (Peterson & Hoagland, 1990; Peterson *et al.*, 1990) and selective herbivory (Tuchman & Stevenson, 1991). Studying the effect of these and other factors such as nutrient limitations, the presence of bacteria and competition with other diatoms might be useful to explain its success in the biofilms.

In a further study, efforts were taken to analyze the chemical composition of various complex forms of EPS which are observed in araphid diatoms. We used freshwater araphid diatoms such as *Fragilaria*, *Pseudostauroopsis*, *Staurosira* and *Punctastriata* display chain-like colonies held together by cell-cell attachments via spines originating from the frustules which interlock two adjacent cells (Williams & Round, 1987). For isolation and characterization of EPS, specific fractionation methods are necessary. Loosely attached EPS is normally extracted by warm water treatment (30 °C) (Staats *et al.*, 1999). EPS structures such as stalks, pads and tubes are not extractable at these conditions. In our study, after warm water extraction, the presence of frustule associated EPS and intercellular pads was observed by staining with alcian blue (Daniel *et al.*, 1987). For extraction of such closely associated EPS, we used various methods such as extraction at 20 °C and 30 °C using NaCl, NaOH, EDTA (Staats *et al.*, 1999) and NaHCO<sub>3</sub>. Then we used NaHCO<sub>3</sub> at 95 °C (Wustman *et al.*, 1997) and NaOH at 95 °C (Chiovitti *et al.*, 2003). From these analyses, the last two solutions were found to be useful for extraction of intercellular pads while NaOH treatment showed complete dissolution of the frustules.

Generally, SE is released in to the surrounding medium and it can be extracted by concentration and precipitation. The loosely attached EPS was extracted by using warm water (30 °C) treatment (de Brouwer & Stal, 2002; Staats et al., 2000) followed by hot water (90 °C) (Wustman et al., 1997) treatment which extracts EPS tightly adhered to the cells. Hot carbonate treatment was able to extract most of the intercellular pads and finally hot alkali treatment extracted all possible EPS that was present as pads or closely associated with frustules (Chiovitti et al., 2005). By using this method, we could fractionate and analyze nearly all EPS. This method was used successfully to study monosaccharide composition secreted by *Pseudostauroopsis sp.*, *Staurosira sp.*, *Punctastriata sp.*, *C. microcephala*, *C. minuta*, *A. minutissima* and *Fragilaria capucina*.

These analyses demonstrated the presence of higher amounts of polysaccharides and proteins in the hot carbonate (HB) and hot alkali (HA) fractions. This suggests the involvement of proteins and polysaccharides in the pads and in the frustules. In earlier reports, high amount of proteins were reported in frustule associated EPS of *Pinnularia viridis* (Chiovitti et al., 2003) and tubes of *Berkeleya* (Daniel et al., 1987). The presence of proteoglycans (Lind et al., 1997) and glycoproteins were reported in the gliding trails (Chiovitti et al., 2003) of different diatoms. Silaffin proteins are useful in silica precipitation (Kröger et al., 2001; Kröger et al., 1997). Still exact composition of EPS and frustule formations are mostly unknown. Here we could analyze presence of limited number of monosaccharides and only one uronic acid by HPLC. Broad scale analyses of all sugars, uronic acids, proteins with considering structural aspects will yield valuable information regarding formation of silica wall and EPS structures.

Heterogeneous monosaccharide profiles were observed in *Pseudostauroopsis*, *Staurosira* and *Punctastriata* isolates and were grouped together in PCA, whereas other species (*C. microcephala*, *C. minuta*, *A. minutissima* and *Fragilaria capucina*) showed very distinct monosaccharide signatures. When we compared the results of PCA and the phylogenetic tree of araphid diatoms, *Pseudostauroopsis*, *Staurosira* and *Punctastriata* showed heterogeneity as seen in the PCA of monosaccharides. Therefore, monosaccharide profiles might be a tool for phylogenetic analysis. But before using such profiles for phylogenetic analysis many experiments are necessary to analyze changes in monosaccharide profiles of single strain during nutrient limitations, and chaining temperature and light condition.

### **Diatom bacteria interactions**

Diatoms and bacteria are the un-separable part of epilithic biofilms from the photic zone. Studies on of various interactions between both groups might throw light on formation and changing of community structure. Early colonizing bacteria depends upon available dissolved organic matter from the overlaying water, whereas latter stages bacterial community structures depend upon the composition of autotrophs (Jackson, 2003) and it might be driven by resource utility. Therefore, it becomes a close system (Jackson, 2003). Positive correlation between bacterial abundance and algal attachment (Hodoki, 2005) and the presence of a specific bacterial community might be responsible for the attachment of diatoms to the surface (Gawne et al., 1998). Co-occurrence of satellite bacteria in unialgal cultures has been reported in marine (Schäfer et al., 2002) and freshwater environments (Makk et al., 2003). Common features were observed between these studies and the diatom-associated bacteria from Lake Constance i.e. a dominance of  $\alpha$ -proteobacteria followed by CFB,  $\beta$ - and  $\gamma$ -proteobacteria (Chapter 8). All these bacterial communities are the major players in degradation of soluble and particulate organic matter and amino acids as a sole source of organic carbon (Makk et al., 2003; Rosenstock & Simon, 1993; Schweitzer et al., 2001). Diatom secreted EPS might be the major source for most of heterotrophic bacteria and fast utilization of this EPS has been demonstrated earlier (Giroldo et al., 2003). Diatom-bacterial interaction might be stimulatory, inhibitory symbiotic or parasitic. A stimulatory effect of specific presence of specific bacteria from  $\alpha$ - and /or  $\beta$ -proteobacteria on diatom growth observed (Bruckner, unpublished data), whereas inhibitory effect was also demonstrated (Cole, 1982; Wigglesworth-Cooksey & Cooksey, 2005). Bacteria secreted products such as vitamin B12 might be useful for enhancing growth of diatoms (Croft et al., 2005) inhibitory effect might be due to modification of environment by bacteria or presence of lytic bacteria (Cole, 1982).

Bacteria are not easily cultivable on artificial media and it is well known that only 1% of the total bacteria from a particular environment can be cultivated. To improve the culturability of bacteria various strategies have been designed. The use of dilute media (Watve et al., 2000) and use of signal compounds (e.g. Acetyl homoserine lactone) are some of the successful strategies (Bruns et al., 2003). In our study, we used the spent medium from four different isolates of the diatom genus

*Cymbella*. This medium contained mainly diatom-secreted polysaccharides, at concentration of 20-110  $\mu\text{g/ml}$  and proteins in traces. We found that within 15 days, the bacteria had consumed the majority of the EPS portion and the bacteria. Thus, the substrate in the form of diatom-spent medium was useful for an initial enrichment of bacteria from natural biofilms. One of the dominant bacterium isolated here was distantly related to *Azospirillum*, (91% similarity of 16S rDNA) a well known nitrogen fixer associated with grasses (Peng et al., 2006). Thus, the use of such 'natural medium' proved to be useful for cultivation of bacteria and even novel bacteria. Further experiments to find out which components of EPS / spent medium do they degrade, would be interesting.

## Summary

In this work, various aspects of diatom communities from epilithic biofilms of Lake Constance were studied. The diatom and bacterial community structure and extracellular polymeric substances (EPS) were studied at five nearby locations and at four different depths from epilithic biofilms. This study revealed a high species richness of diatoms and a dominance of  $\beta$ -proteobacteria, *Cytophaga* / *Flavobacteria* / *Bacteroides* (CFB) group and high GC containing gram-positive bacteria. Higher amount of EPS was observed in the samples from lower depth. Seasonal fluctuations of the diatom community and concentrations of soluble (SE) and bound EPS (BE) were studied from epilithic biofilms collected during June 2004-June 2005. The higher diversity and also higher EPS content was observed in biofilms from April 2005. We tried to cultivate dominant as well as rarely found diatom species from the biofilms and identified then by classical methods based on morphological characters. More than 100 isolates were cultivated belonging to 20 different genera and 44 species. The 18S rDNA region was sequenced from 55 diatom isolates and phylogenetic trees were constructed. They revealed a clear separation within raphid and araphid diatoms. Axenic diatoms were grown for 28 days under identical growth conditions (temperature, light intensity, nutrients) and their growth behavior, patterns of EPS secretion and the corresponding monosaccharide profiles were studied. As in this experiment we observed that a major portion of BE was remained un-extractable, various published methods were tried and finally an optimized protocol was used for the fractionation of all EPS secreted by diatoms from various genera. FITC labeled-lectins, DAPI and DTAF were used for localization of various EPS structures such as tubes, stalks, capsules and pads. As diatoms and bacteria are the dominant members in the biofilms, uni-algal diatom cultures were studied for the associated bacteria using 16S rDNA clone library approach. The spent medium from diatoms was inoculated with an epilithic bacterial community in a dilution series to explore the utilization of EPS, a natural substrate, for the growth of bacteria. One of the dominant bacterial strain was isolated found to represent a new taxon.

## Zusammenfassung

Im Rahmen dieser Arbeit wurden Kieselalpengesellschaften der epilithischen Biofilme des Bodensees untersucht. Die Lebensgemeinschaften von Diatomeen und Bakterien und deren extrazelluläre lösliche Substanzen (EPS) wurden an fünf verschiedenen benachbarten Probennameorten für vier verschiedene Tiefen charakterisiert. Eine große Artenvielfalt bezüglich der Diatomeen wurde vorgefunden. Die dominanten Bakteriengruppen in diesen Proben waren  *$\beta$ -Proteo-*, *CFB-* und grampositive Bakterien, letztere mit hohem GC-Gehalt. In Proben aus geringeren Tiefen wurden höhere EPS-Konzentrationen vorgefunden, ferner annuelle Fluktuationen bezüglich löslicher und gebundener EPS von Juni 2004 bis Juni 2005. Es wurde versucht häufig wie auch selten vorgefundene Kieselalgen zu kultivieren. Die Klassifizierung der Arten geschah anhand morphologischer Merkmale. Es wurden über 100 Stämme isoliert, die 44 Arten und 20 Genera zugeordnet werden konnten. Von 55 Isolaten wurde die 18S rDNS sequenziert und phylogenetische Stammbäume erstellt. Dabei konnten raphide Diatomeen klar von araphiden abgegrenzt werden.

Bei identischen Wachstumsbedingungen (Temperatur, Lichtstärke, Nährstoffe) wurden die EPS Sekretion hinsichtlich der Monosaccharid-Zusammensetzung untersucht. Das Verfahren wurde anhand verschiedener, in der Literatur vorhandener Methoden für die kultivierten Diatomeen optimiert. FITC-gekoppelte Lektine, DAPI und DTAF wurden zur Lokalisation verschiedener EPS-Strukturen wie Schläuche, Stiele, Kapseln und Pads verwendet. Da Diatomeen und Bakterien Biofilme dominieren wurden kontaminierte Kulturen bezüglich der bakteriellen Artenzusammensetzung mittels 16S rDNS Bibliotheken untersucht. Kulturüberstand von Diatomeen wurde mit Bodensee-Bakteriengemischen über Verdünnungsreihen inokuliert um den EPS-Abbau durch letztere zu untersuchen. Eine der dominanten Bakterienarten wurde dabei isoliert und repräsentiert ein neues Taxon.

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## **Contributions**

All work has been done by me or under my supervision unless stated. This work was carried out from April 2003 and December 2006 under able guidance of Prof. Kroth.

Chapter 4      Linda Medlin contributed by identifying diatom isolates, SEM and guidance for phylogentic analysis. Prof. Mendgen performed SEM and photographed some of diatom isolates.

Chapter 8      Monali Rahalkar helped me in designing experiments, writing manuscript, and half of the work done in experiment about utilization of EPS and in isolation of bacteria. Christian Bruckner did half of the 16S rDNA clone libraries and sequencing of unique clones from them. Prof. Schink helped us in designing the experiments and gave valuable suggestions.