Happiness and bacteria have one thing in common:
they multiply by dividing.

~Rutvik Oza

Role and function of hydrolytic enzymes for cycling of organic material within microbial mats

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Abstract

Wind flats are found along the coastline of the southern Baltic Sea. Microbial mats are the first to colonize these nutrient-poor habitats. Microbial mats are an entire ecosystem with the main functional groups cyanobacteria, diatoms, heterotrophic bacteria, purple sulphur bacteria and sulphate reducing bacteria. Organic material is produced and degraded within small temporal and spatial scales.

This thesis aims to characterise the wind flat microbial mat as habitat for heterotrophic bacteria in contrast to microbial mats from tidal flats (1), to evaluate the importance and efficiency of hydrolytic enzymes to degrade the organic material within the microbial mat (2) and finally to assess the ability of the heterotrophic bacteria microbial mat community to adapt themselves to changing organic material load (3).

The lacking regular mechanical disturbances by tides within the wind flat have influence on the microbial mat community: microbial mats established permanently within the wind flat, but did not feature steep gradients and a clear separation of the functional groups like in tidal microbial mats. Especially strictly anaerobic bacterial groups (purple sulphur bacteria and sulphate reducing bacteria) are not permanently present in the wind flat microbial mat.

Hydrolytic enzymes are not of major importance for the heterotrophic bacteria to gain substrates and nutrients. They predominantly assimilate free and dissolved organic material with low molecular weight which is present in surplus within the microbial mat. Nevertheless, heterotrophic bacteria are able to utilize complex C-compounds and are not only adapted to the easily accessible compounds provided by the primary producers

Right now, the organic material is accumulating within the microbial mat due to very slow and ineffective turnover by the hydrolytic enzymes. This leads to the natural succession and degrading of the microbial mat. The salt marsh community with *Puccinella maritima* (Hudson) Parlatore (common salt marsh grass) could established itself more and more over the years. Thus, the silting-up process within the wind flat is proceeding. The examined microbial mat may vanish from its actual location and colonize an until now mat-free area of the wind flat.

Contents

List of abbreviations

α-ABA	α-amino butyric acid
α-Gluc.	α-glucosidase
Ala	Alanine
AMC	7-Amino-4-Methylcoumarin
AP	Aminopeptidase
Arg	Arginine
Asn	Asparagine
β-Gluc.	β-glucosidase
Asp	Aspartic acid
b.p	before present
bp	basepair
BSA	bovine serum albumin
Cellob.	cellobiodase
Cys	Cysteine
DAPI	4', 6-diamidino-2phenyl indole
DCAA	dissolved combined amino acids
DFAA	dissolved free amino acids
DMSO	dimethyl sulfoxide
DOC	dissolved organic carbon
DOM	dissolved organic material
DIN	dissolved inorganic nitrogen
DON	dissolved organic nitrogen
DM	dry mass
EC	enzyme commission
EPS	extracellular polymeric substances
FM	fresh mass
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histedine
HPLC	high pressure liquid chromatography
Iso	Isoleucine
Leu	Leucine
Lys	Lysine

Met	Methionine
MUF	4- Methylumbelliferon
NAC	N-Acetylglucosaminidase
Orn	Ornithine
PAA	particulate amino acids
Phe	Phenylalanine
Pro	Proline
Ser	Serine
SRP	soluble reactive phosphor
SYP	Seawater Yeast Peptone
TAA	total amino acids
Tau	Taurine
THAA	total hydrolysable amino acids
THF	Tetrahydrofuran
Thr	Threonine
Tris	Tris(hydroxymethyl)-aminomethan
Trp	Tryptophane
Tyr	Tyrosine
Val	Valine

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1. Introduction

The Baltic Sea is totally surrounded by land with a catchment area of ~ 1,700,000 km^2 with a population of 85 million inhabitants (HELCOM, 2009). Anthropogenic pressure on this area is immense. Eutrophication is one of the most severe threats for the Baltic Sea as it changes the structure and functioning of the entire marine ecosystem and leads to a reduction in ecosystem stability (HELCOM, 2009). Indications for eutrophication are: increasing concentrations of dissolved nutrients (N and P), organic carbon and a high productivity (100 – 500 g C m^{-2} y^{-1} for benthic communities) (Meyer-Reil and Köster, 2000). Especially coastal regions as interface between land and sea with a limited water exchange are mostly affected (ibid.). They play an important role for production and remineralisation of organic matter (Cook et al., 2004a). They can be seen as specific cleaning filters (Brown and McLachlan, 2006) and about 70 % of organic matter reaching a marine beach is mineralized by bacteria and converted into their own biomass (Koop and Griffiths 1982).

The Baltic Sea is a sea without a tidel hub. Hence, no tidal but so-called wind flats are present. Water level, wind speed and direction determine if the flat is flooded or not. Wind flats are typical for the southern Baltic Sea. Large parts of the flats are covered by microbial mats. Microbial mats can be defined as complex biofilms characterised by physical and chemical gradients (light, O_2, H_2S) and stratification of organisms in different layers where they find their favourable conditions (Stolz, 2000). Microbial mats are founded by cyanobacteria colonising habitats, like tidal flats with extreme fluctuations in salinity, light, temperature etc. and low amounts of nutrients (Karsten and Kühl, 1996). These pioneer colonisers enrich the sediment with organic material and other microorganisms can accumulate (Stal, 1994, van Gemerden, 1993). Microbial mats develop within environments where benthic meio- and macrofauna is hampered and grazing is restricted (Walter, 1976; Jorgensen et al., 1983). Burrowing organism (bioturbation) would destroy the physico-chemical gradients within the microbial mats (Fenchel, 1998).

Their fossil counterparts, stromatolites, found in Western Australia and South Africa could be dated back to 3500 million years b.p. and are the oldest records for photosynthesis evolution (Awramik, 1992). They have significantly influenced the composition of the atmosphere with O_2, H_2 and CH_4 (Hoehler et al., 2001). Microbial mats can be used as a model system for understanding the development of earth's oxygenic atmosphere (McNamara and Awramik, 1992).

In coastal areas, microbial mats stabilise the sediment and protect the coastline. Several characteristics lead to this biogenic stabilisation: the dense network of the cyanobacterial and bacterial filaments, which additionally produce extracellular polymeric substances (EPS) forming a sticky matrix entrapping particles (Paterson, 1994). Sediment can be stabilised by microbial mats by the 5 to 21 – fold (Paterson, 1994; van Gemerden, 1993; Yallop, 1994).

Productivity and organic material turnover within microbial mats are functionally comparable with those of planktonic ecosystems. However, microbial mats are 10^3 – 10^5 –times as active as the organisms are concentrated in much smaller areas (Jorgensen, 1994). Cyanobacteria as the dominating primary producers (Garcia-Pichel et al., 1996) have low growth rates but a high primary production within microbial mats (Guerrero and Mas, 1989). Thus, the majority of photosynthetically fixed carbon must be quickly recycled, when both low growth rates and high primary production are present (Ludwig et al., 2006).

Lipid characterization within a hypersaline microbial mat displayed high amounts of stanols and stanones indicating extensive microbial degradation processes (Rontani and Volkman, 2005). Microbial mats are called "the world's smallest ecosystem" because production and remineralisation of organic material take place within small temporal and spatial scales (Karsten and Kühl, 1996). Thus, they are a good system to study and understand the cycling of nutrients and organic matter (Visscher and Stolz, 2005).

The pathways of nitrogen into and within the microbial mats, for instance, are well studied: A rapid incorporation of dissolved inorganic nitrogen (DIN) into biomass and an effective cycling of nitrogen occur in oligotrophic ecosystems (Cook et al., 2004b). Nitrogen can also be recruited from atmospheric N_2 via nitrogen fixation. Within tidal microbial mats, the mat location within the flat and hence frequency of flooding indirectly influences the amount of fixed atmospheric nitrogen. The higher the mechanical forces the more nitrogen is gained via nitrogen fixation to compensate the losses of organic material washed out by the tides (Bebout et al., 1994). Denitrification and nitrification are negligible as these rates are often very low and/ or even below the detection limit (Nielsen and Sloth, 1994; Bonin and Michotey, 2006; Ludwig et al., 2006). Dissolved organic nitrogen (DON) is the dominating nitrogen-compound released in areas with high organic inputs by primary producers (Cook et al., 2004b; Tyler et al., 2001; Eyre and Ferguson, 2002).

Microbial mats are the pioneer-settlers in "dessert-like" habitats. When nutrients accumulate, higher plants can establish (van Gemerdern, 1993). The addition of phosphorous into a salt-marsh of northern Belize lead to a colonization by the macrophytes *Eleochans cellulosa* and *E. interstincta.* The microbial mats in this area vanished due to these macrophytes (Sirova et al., 2006). A silting-up progress was observed also in the examined wind flat. First individuals of *Puccinella maritima* (Hudson) Parlatore (common salt marsh grass), a typical pioneer in coastal regions were observed in 2002 (Woelfel et al., 2007). Over the years, a more and more stable population could establish within the area (Heyl et al., 2010). According to sucsessional stages, this wind flat now seems to be in a transitional state between the microbial mat and the salt marsh community. So on the one hand, the natural silting-up progress and on the other hand, the proceeding eutrophication of the Baltic Sea are the driving forces for the further development of the microbial mats within the wind flat.

The microbial mat community is comprised of four major functional groups: oxygenic phototrophs (cyanobacteria, diatoms), aerobic heterotrophic bacteria, sulphate oxidising bacteria and sulphate reducing bacteria (Visscher and Stolz, 2005). Diversity and complexity are the most significant features of a microbial mat. The phototrophic layer within microbial mats is clearly dominated by cyanobacteria. However, phylogenetic analyses reveal a clear dominance and diversity of heterotrophic bacteria (Ley et al., 2006). This heterotophic bacterial diversity indicates the possible complexity of metabolic pathways for organic material degradation and nutrient recycling within microbial mats. Until now, the role of heterotrophic bacteria for organic material cycling is poorly studied in microbial mats.

Different bacteria groups show different metabolic profiles. The Bacteriodetes-group consumes refractory high molecular weight compounds and the α-Proteobacteria mostly utilize labile low molecular weight compounds (Cottrell and Kirchman, 2000). Therefore, the dominance of α-Proteobacteria in the photorophic layer within wind flat microbial mats may indicate a better availability of already utilizable or easy degradable substrates (Heyl, 2007). The organic material produced by primary producers in microbial mats is mainly delivered as exudates (Underwood et al., 1995). Primary producers, their activity (quantity of organic material) and physiological status (quality of exudates) determine the bacterial community (Jensen, 1983; Cook et al., 2004c).

Prokaryotes are called "the unseen majority" because they account for up to $1.18 \cdot 10^{29}$ cells with a carbon content of $305 \cdot 10^{15}$ g C in aquatic habitats (Whitman et al., 1998). This masses compare to 60 – 100 % of the carbon found in plants (ibid.). Bacteria and Archae transform dissolved organic carbon (DOC) into particulate matter (bacterial biomass production) or to CO_2 (bacterial respiration) (Chorst et al., 1986). Bacteria benefit from their large surface : volume - ratio and are very efficient in assimilation and utilisation of surrounding substrates (Bratbak and Thingstad, 1985). They show the highest metabolic rates per biomass unit (Chrost et al., 1986). Heterotrophic bacteria rely on organic material as their major energy source, in contrast to phytoplankton/ -benthos depending on light and inorganic nutrients or zooplankton/ -benthos feeding mainly on bacteria and/ or detritus.

Microorganisms, especially bacteria, are important for cycling nutrients within aquatic ecosystems. The microbial loop concept adds another bacterial function to the traditional remineraliser function: bacteria are assimilators of released dissolved organic material (DOM) by phytoplankton (Azam et al., 1983). Microorganisms (bacteria and protists) form their own food web connected to the classical food web (Hoppe, 2002).

Furthermore, bacterial secondary production is often comparable to the primary production by phytoplankton (Chrost, 1992). Especially oligotrophic aquatic ecosystems, tend to be net-

heterotrophic as bacterial respiration exceeds primary production (Cole, 1999; Cotner and Biddanda, 2002; Lennon and Pfaff, 2005).

Microbial biomasses and degradation activities increase with the organic carbon supply. At substrate saturation, biomasses and activities are uncoupled from DOM and other parameters like oxygen become determining for degradation efficiency (Köster et al., 1997). Apart from the obvious higher microbial activity with increasing nutrient/ organic matter loads (Taylor et al., 2003; Chrost et al., 1986), less is know if higher microbial activities actually can compensate the higher organic load.

More than 95 % of the DOM in aquatic ecosystems consists of polymeric compounds, which have to be broken up before bacteria can assimilate them (Chrost, 1991). Bacteria are responsible for the degradation of DOM within aquatic ecosystems by hydrolytic enzymes production (ibid.). Hydrolytic enzymes catalyse the hydrolysis between compounds by using water. They can be divided into several subclasses and are sorted by EC (Enzyme Commission) - numbers in a database: esterases (cleaving ester bonds; EC 3.1.1.xx), phosphatases (recruitment of soluble reactive phosphate; EC 3.1.3.xx), glycusidases (carbohydrate degradation; EC 3.2.1.xx) and proteases (breaking up proteins and/or peptides; EC 3.4.11.xx) (Brenda-enzymes.org 26.07.2011).

There is a discrepancy between classical enzymology and ecological field studies in terms of enzymes. Both speak about enzyme activities, e.g. α-glucosidase activity. A classic enzymologist refers with the term "enzyme activity" to one enzyme, catalogued by an EC - number in a database. Enzymes are described by the reaction they catalyse, the occurring products and the specificity for substrates etc.. An ecologist refers to "hydrolysis velocity" when speaking about enzyme activities (Hoppe, 1983). Strictly speaking, the obtained enzyme activities in ecological field studies, like α-glucosidase activities, should be addressed as "D-glucose hydrolysed from α-glycosidic bondages". Anyway, the hydrolytic activities are addressed as activity of α-glucosidase etc. for better understanding and readability. The analysed hydrolytic enzyme activities in this study are listed and connected with database entries of hydrolytic enzymes and their catalysed reactions and specificities to get a better understanding about the enzymatic reactions catalysed (Tab.1.1)

It is almost impossible to determine which species actually contributed to the total hydrolytic activity in natural communities. Therefore, all obtained turnover rates and the derived kinetic parameters have to be addressed as apparent enzymatic activity, because the hydrolysis rate is not the result of a single enzyme from one organism or species. Bacteria are the main hydrolytic enzyme producers and possess many different types (Martinez et al., 1996). Investigations on hydrolytic enzymes within phytoplankton/ -benthos or zooplankton/ -benthos are scarce until now, but they can produce hydrolytic enzymes as well. For instance, algae produce alkaline phosphatase to cover their phosphate demand when soluble reactive phosphate (SRP) is limited (Quisel et al., 1996).

Hydrolytic enzymes acting outside the cell are the most relevant ones. Their activity is the limiting and determining factor for degradation of organic material (Misic and Harriague, 2007). Hydrolytic enzyme activities are a good parameter to evaluate the ecosystem status (Kierstyn et al., 2001). Bacteria can respond rapidly to changing environmental conditions (Hayes, 2008). They regulate their enzymes according to the changing availability of nutrients faster than they build up new biomass or the community composition changes (Sala et al., 2001). The release of low molecular weight compounds by hydrolytic enzymes may be the rate-limiting step for bacterial production (Hoppe et al., 1998) Bacteria are limited by the rate of substrate supply but at the same time they are also responsible for the actual substrate concentration (Hoppe et al., 1988). Thus, the potential of a community to degrade organic material is assessed. Furthermore, the relative enzyme activities indirectly reflect the prevailing substrate composition (Köster et al., 1997; Chrost et al. (1986). The hydrolytic enzyme activities evaluate which classes of macromolecules are important and accessible to the bacterial nutrition (Taylor et al., 2003; Sala et al., 2001).

The fluorogenic model substrates for extracellular hydrolytic enzyme activities presented by Hoppe (1983) are restricted to enzymes cleaving the terminal monomers. These kinetics are easier to study and the reactions produce utilisable products (Sinsabaugh et al., 2008). Most ecological studies do not examine endohydrolytic enzymes cleaving from the polymeric compound which results in oligomers. Thus, information is incomplete how endohydrolytic enzymes degrade highmolecular polymeric compounds are incomplete (Ziervogel and Arnosti, 2008). On the other hand, N-terminal aminopeptidases are the dominating proteolytic enzymes within aquatic environments (Billen, 1991).

The polymeric compounds in marine ecosystems include proteins, starch, lipids, pectin, cellulose, chitin, nucleic acids and phospholipids (Arnosti et al., 1998; Martinez and Azam, 1993). The structural heterogeneity of biopolymers requires the interaction of several enzyme classes to break them down to the utilisable monomers available for microbial consumption (Sinsabaugh et al., 2008). The molecular size of the substrates determines their turnover time, i.e. short-chain lipids are faster degraded than long-chained ones, unsaturated bonds are more reactive than saturated ones (Wakeham and Canuel, 2006). The ability to degrade certain polymeric compounds is divers among bacteria. Many stems are able to degrade starch, whereas only a few can break down chitin or cellulose (Podgorska and Mudryk, 2003).

Tab.1.1: Overview about database entries (BRENDA and MEROPS) of hydrolytic enzymes which are comparable with the hydrolytic activities measured in this study: EC (enzyme Comission) number, systematic name, reaction catalyzed with substrate specifity (if known) and how the corresponding hydrolytic activity is named in this study. X- = amino acid; X-Y- = dipeptide

Named in this study	Corresponding EC number	Systematic name	Reaction catalysed
Ala-aminopeptidase	3.4.11.2	Membrane alanyl aminopeptidase	Release of N-terminal amino acids. Released as X- or X-Y- from a peptide, amide or arylamide. X is preferably Alanine (Ala), but may be most amino acids including Proline (Pro) (slow action). When a terminal hydrophobic residue is followed by a Pro-residue, the two may be released as an intact X-Pro dipeptide.
Arg-Aminopeptidase	3.4.11.6	Aminopeptidase B	Release of N-terminal Arginine (Arg) and Lysine (Lys) from oligopeptides when P1' (first amino acid next to the cleavage position in direction to N-terminus) is not Pro. Also acts an arylamides of Arg and Lys.
Leu-aminopeptidase	3.4.11.1	Leucyl aminopeptidase	Release of an N-terminal amino acids as X or X-Y-. X is preferably Leu, but may be other amino acids including Pro (except Arg or Lys).Y may be Pro. Amino acids amides and methyl esters are also hydrolysed but rates on arylamides are exceedingly low.
Phe-aminopeptidase	No entries found		
α-glucosidase	3.2.1.20	α-D-glucoside glucohydrolase	Specifity for exohydrolysis of 1,4-α-glucosidic linkages. Oligosaccharides are rapidly hydrolysed relative to polysaccharides which are hydrolysed relatively slow, or not at all.
β-glucosidase	3.2.1.21	β—D-glucoside glucohydrolase	Wide speciftiy for β-D-glucosides. Some examples also hydrolyse one or more of the following: β-D-galactosides, α-L-arabinosides, β-D-xylosides, β- D-fucosides
cellobiodase	3.2.1.91	Cellulose 1,4-β-cellobiosidase	Hydrolysis of 1,4-β-D-glucosidic linkage in cellulose and cellotetraose, releasing cellobiose from the non-reducing end of the chains.
N-Acetylglucosaminidase	3.2.1.52	β-N-Acetylhexoseaminidase	Acts on N-Acetylglucosides and N-Acetylgalactosides

1.1 Hypotheses

The main aim of this study was to assess the status of the microbial mat in the wind flat in terms of accumulating or degrading organic material. Carbohydrate and protein degradation within wind flat microbial mats are analysed. Therefore, following hypotheses will be addressed:

1. The wind flat microbial mat presents a stable habitat because of lacking tides. The wind flat microbial mat community is not as much affected by abiotic changes as other communities in microbial mats or sediments.
2. Microbial mats are described to colonise extreme habitats. The wind flat microbial mat itself is not an extreme habitat. Heterotrophic bacteria find a sufficient substrate supply and benefit from easily accessible exudates by the primary producers. Hence, the microbial mat tends to accumulate organic material.
3. Heterotrophic bacteria find a sufficient supply of easily accessible substrates within the microbial mat. They do not feature a great metabolic flexibility as the wind flat microbial mat is not characterised by steep gradients and ever changing abiotic parameters compared with other micobial mats and coastal sediments. Especially their ability to degrade high molecular compounds should be limited.

2. Material and methods

2.1 Field work

2.1.1 Wind flat "Bock"

The studied microbial mat is located in the wind flat "Bock", the eastern tip of the peninsula Fischland-Darß-Zingst (Fig. 2.1). The area extends from N 54° 28.3' to N 54° 26.48' in north-south direction and from E 012° 54.6' to E 013° 3.4' in west-east direction. It covers an area of 1600 hectare and the average water level is 5 – 25 cm (Reinhard, 1953). Tideless wind flats are typical for the Baltic Sea coast in contrast to tidal flats at the North Sea coast. They are exposed and irregularly flooded only in dependence of water levels and the prevailing wind speed and direction. The peninsula Fischland-Darß-Zingst evolved with the thawing of the Weichselian ice sheet after the last glaciation (15,000 y before present (b.p.)) during the Littorina transgression (8,000 y b.p.) (Reinhard, 1953). The coastline changes continuously due to steadily eroding material, which is deposited at other places (Schwarzer, 1996). This erosion is triggered by east to west going currents parallel to the coastline. The wind flat "Bock" is the youngest formation of the peninsula and developed due to natural processes (sediment transport by currents) and human activities: sediment was dredged to secure fairways for shipping traffic and to protect the coast. Additionally, the area was afforested to support coastal protection (Reinhard, 1953).
Nowadays, the wind flat "Bock" is part of the national park "Vorpommersche-Boddenlandschaft" which encompasses a total area of 805 km^2 with 687 km^2 water area.

2.1.2 Sampling

Microbial mat samples were taken monthly over a period of 15 months during 2008 – 2009 with 13 samplings at N 54° 26.71' E 012° 55.79'. Samples were taken with a clean Petri dish (92 mm diameter; 16 mm height; polystyrol, Sarstedt), sealed with parafilm and transported dark and cooled to the laboratory within 3 – 4 h. If necessary, the samples were stored at about 15 °C (Liebherr-Vinothek) and 10 – 20 µmol photons m^{-2} s^{-1} with a 16 h : 8 h light : dark light regime (OSRAM, Lumilux de Luxe Daylight) and were analysed within the following day.
The first two samplings were used to evaluate and improve sampling and storage conditions. The Petri dish is the unit from which all subsamples were taken to analyse all addressed parameters (biomasses, organic material, hydrolytic enzyme activities etc.). Hence, the heterogeneity within the Petri dish should not be too high (spatial side effects). Storage of samples for a longer time could also have an effect (temporal side effects). Sediment subsamples (squares of 1 · 1 cm) were carved out of a single Petri dish. Chlorophyll *a* and bacterial abundance were determined alternating in every second subsample to address the spatial heterogeneity (August 2008).

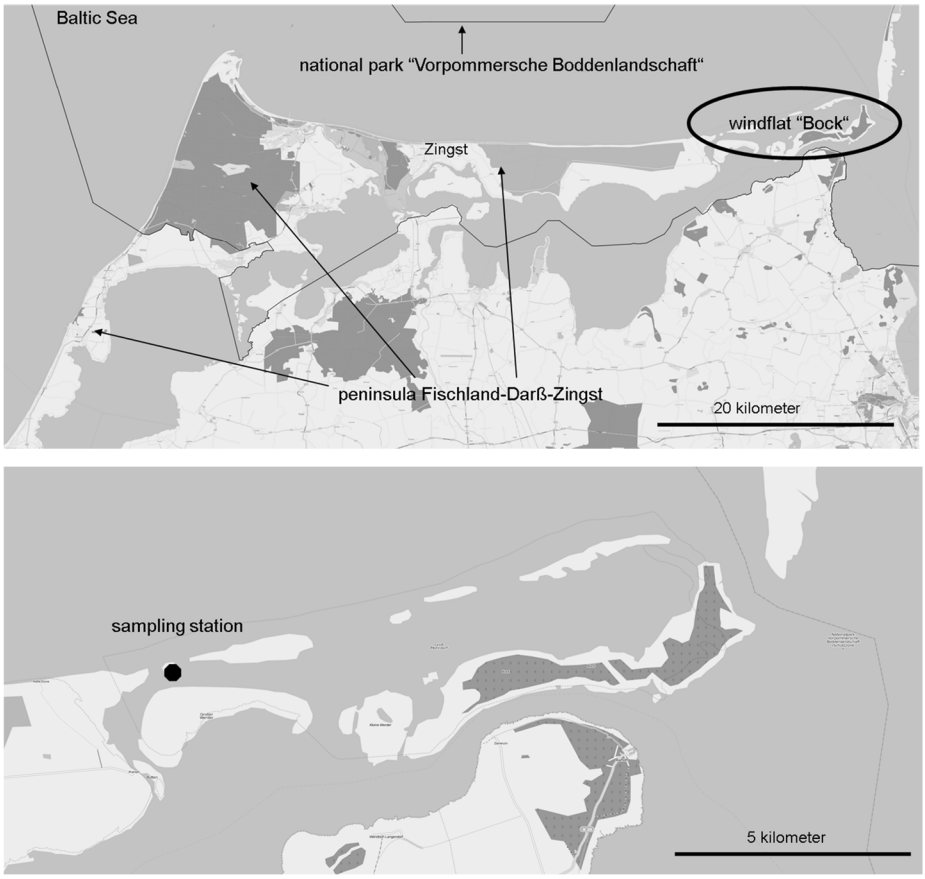


Fig. 2.1: Geographical location of the sampling site (black dot), located in the windflat "Bock" (black circle) at the eastern end of the peninsula Fischland-Darß-Zingst in the national park "Vorpommersche Boddenlandschaft (black line). Source: openseamap.de, modified

One Petri dish from September 2008 was stored as described above for six days and chlorophyll *a* and bacterial abundance were determined every day to assess potential storage effects (Procedure of biomass determination s. 2.1.5).

Bacterial abundances ranged between 1.7 – 3.5 cells · 10^9 cm^{-3} (median 2.7 cells · 10^9 cm^{-3}) both in time and in space (Fig. 2.2 A, B). Chlorophyll *a* was with 27 – 85 µg cm^{-3} (median 41 µg cm^{-3}) on the spatial scale about 2 – 4 times lower compared to 48 – 245 µg cm^{-3} (median 132 µg cm^{-3}) on time scale. Values were normalised by the corresponding median to compare spatial and temporal heterogeneity. Both Box-Whisker plots were of the same width (Fig. 2.2 C, D). Hence, spatial and temporal heterogeneity were on the same level. The Petri dish as smallest subunit for sampling as well as the storage conditions is sufficient.

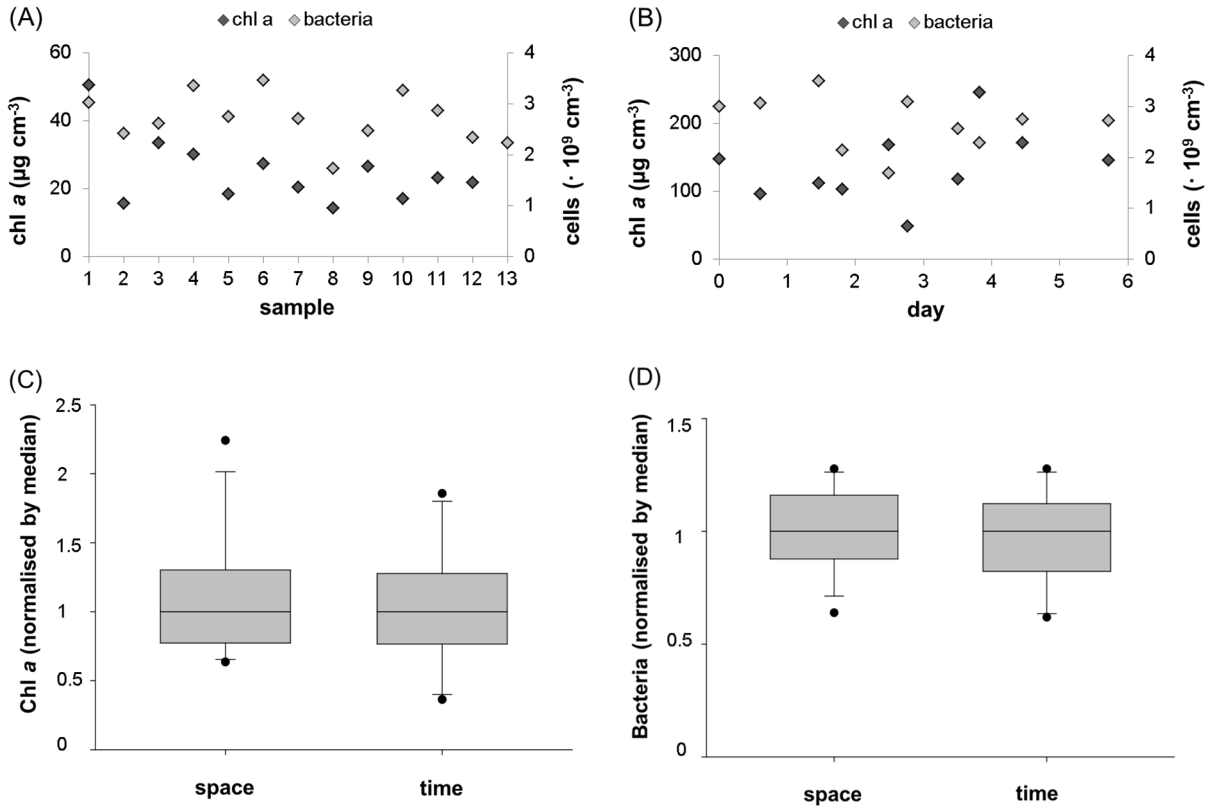


Fig. 2.2: Biomass heterogeneity within one petri dish for chlorophyll *a* in µg cm^{-3} and bacterial abundance in cells · 10^9 cm^{-3} examined for the whole petri dish (space) (A) and one sample of one petri dish every day for one week (time) (B) (n = 1). Box-Whisker-plots giving median, 25 - % and 75 % - quartile, 95 % - quartile and extreme values for (C) chlorophyll *a* normalized by median (n = 12 (space) resp. 10 (time)) and bacterial abundance normalized by median (n = 13 (space) resp. 10 (time)).

Biomasses and sediment characteristics were determined at every sampling. Kinetics for hydrolytic activities were performed three times for glycolytic resp. proteolytic activity. Different focuses were set at each sampling: depth profiles, utilisation of different carbon sources, organic material analysis and heterogeneity of hydrolytic activities (Tab. 2.1). Thus, number of samples was different for the different parameters at each sampling.

Tab.2.1: Time schedule of examined parameters during the years 2008 and 2009. Number of sample replicates in brackets.

Year	2008		2009									
Parameter	**Aug**	**Sep**	**Jan**	**Feb**	**Mar**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**
Conditions of taking samples/ Method exploration	X (12/ 13)	X (10)										
Biomasses			X (3)	X (3)	X (3)	X (3)	X (3)	X (3)	X (10)	X (10)	X (10)	X (10)
Sediment characteristica			X (3)	X (3)	X (3)	X (3)	X (3)	X (3)	X (10)	X (10)	X (10)	X (10)
Depth profile (Biomasses & enzyme activities)						X (3)			X (3)			X (3)
Kinetics of amino-peptidases (4)					X (3)			X (1)			X (10)	
Kinetics of glycosidases (4)				X (2)			X (1)			X (10)		
Live-dead staining of bacteria						X (3)			X (3)			X (3)
Utilization of different carbon sources						X (3)			X (3)			X (3)
Analytics of amino acids (protein)											X (10)	
Heterogenity						X (32/ 33)			X (10)	X (10)	X (10)	X (10)

2.1.3 Sediment parameters

Sediment fresh mass (FM), dry mass (DM), water content and organic content were examined (as cited in Schlungbaum, 1979). 2 – 5 cm^{-3} sediment was cut out with a plastic syringe, from which the pike was cut-off (radius 1 cm, cylindrical height was 0.75 – 1.35 cm; B Braun). Three samples with four replicates were examined every month. When more then three samples were taken in one month, the rest of the parallels were examined with two replicates each.

The sediment (FM) was weighed in fireproof crucibles (OmniLab) (balance: Sartorius BP 310S). The sediment was dried at 100 °C in an oven (VEB ML W 116-100) over night. After measuring the remaining material, DM and water content were calculated:

$$DM(\%) = \frac{DM(g) \cdot 100\%}{FM} \qquad H_2O - content(\%) = 100 - DM(\%)$$

The samples were combusted afterwards at 550 °C for 4 h in a muffle furnace (Nabertherm B150). The resulting weight delivers the annealing residue (inorganic content of dry weight) and the annealing loss (organic content of dry weight):

$$annealing\_residue(\%) = \frac{Ash_{550°C}(g) \cdot 100}{DM(g)}$$
$$annealing\_loss(\%) = 100 - annealing\_residue(\%)$$

The organic content was transferred into organic carbon, assuming 50 % of the organic mass to be organic carbon.

2.1.4 Organic Material

The organic material pool is diverse in substance classes, composition, complexity, size and quality of the organic compounds. Amino acids were analysed to calculate in detail, how effective the amino acid pool is degraded by hydrolytic enzymes (s. Chap. 4.0.2 and appendix). The amino acids belong to the following fractions:

1. free and dissolved fraction, consisting of monomers (eventually dimers) which can be taken up directly by organisms (dissolved free amino acids = DFAA),
2. dissolved and combined fraction, consisting of compounds ≥ dimers, which mostly have to be broken down enzymatically before uptake and metabolisation by the cell (dissolved combined amino acids = DCAA),
3. particulate fraction, consisting of compounds which have to be broken down enzymatically before uptake. The compounds are either attached to particles/ sediment or bound in biomass so that their bioavailability may be lowered (particulate amino acids = PAA),
4. sum of these three fractions are the total amino acids (TAA). This fraction is called total hydrolysable amino acids (THAA), because amino acids have to be hydrolysed before analysis.

Only DFAA and the THAA could be measured directly due to methodological restrictions. DCAA and PAA can be calculated by subtracting the free and dissolved fraction from the total hydrolysable fraction. It was not possible to distinguish DCAA and PAA from each other.

The dried sediment was stored at -20 °C in plastic vials until further investigation. All used glass devices for extraction and analysis were combusted for 4 h at 550° C to remove residues of organic carbon.

The dried sediment was plounded and 0.5 g of the sediment was weighed into a 10 ml glass tube. The sample was suspended with 2 ml ultrapure water and the DFAA was dissolved by gentle shaking (150 rpm, Johann Otto GmbH KS-15) for 30 minutes (following the protocol for eluting

plant nutrients from sediment by Woelfel et al., 2007). 1 ml of the supernatant was transferred into a HPLC-Vial (Agilent Technologies) and stored at -20 °C until further analysis.

THAA were hydrolysed under acidic conditions. 0.1 g of dried sediment was weighed into a glass ampoule, 2 ml of 30 % HCl and 50 µl of ascorbic acid were added. The samples were fumigated with N_2 to avoid oxidation. All ampoules were closed airtight by melting and incubated for 22 h at 110 °C (Heraeus Instruments T6060). After incubation, 4 ml of 32 % NaOH containing HBO_3 (0.45 mol l^{-1}) were added for neutralisation. The pH was adjusted to 8.3 – 8.7 using 30 % HCl or 32 % NaOH (Knick pH-Meter 765 Calimatic) (Hubberten et al., 1994). The hydrolysates were stored in HPLC-Vials at -20 °C.

Three replicates were extracted for every sample. Amino acids were seperated with high performance liquid chromatography (HPLC). Amino acids were detected via a precolumn fluorescence derivatization with *o*-phthaldialdehyde which binds to free aminogroups and is detected by fluorescence (excitation 340 nm, emission 455 nm). Amino acids were separated via a linear gradient on a Hypersorb ODS 250-RP-C18-column (4 mm I.D., particle size 5 µm) protected with a Hypersorb ODS 120-C18-cartridge (5 x 4 mm, particle size 5 µm). The mobile phase was composed of Methanol : H_2O 9 : 1 (Eluent A) and 0.1 mol l^{-1} phosphate buffer (pH 6.8) with 1.25 vol. % Tedrahydrofuran (THF) (Eluent B) with a flow rate of 0.8 ml min^{-1}. With a commercial standard (Pierce) following amino acids can be detected: L-Alanine (Ala), L-Arginine (Arg), L-Aspartic acid (Asp), L-Cystine (Cys), L-Glutamic acid (Glu), Glycine (Gly), L-Histidine (His), L-Isoleucine (Iso), L-Leucine (Leu), L-Lysine (Lys), L-Methionine (Met), L-Phenylalanine (Phe), L-Proline (Pro), L-Serine (Ser), L-Threonine (Thr), L-Tyrosine (Tyr), L-Valine (Val). The amino acids L-Asparagine (Asn), L-Glutamine (Gln), L-Tryptophane (Trp) and the amino acids degradation products L- Taurin (Tau) (degradation product of Cysteine and Methionine) and L-Ornithine (Orn) (degradation product of Arginine) were added to the commercial standards separately. Standard concentrations of 50 nmol l^{-1} for DFAA and 1.25 µmol l^{-1} for THAA were applied. Additionally, α - amino butyric acid (α-ABA) was used as an internal standard for the detection efficiency of every single amino acid. It was added to every standard and sample in a concentration of 50 nmol l^{-1} (DFAA) or 1.25 µmol l^{-1} (THAA). The amino acids were recognised by their retention times and quantified manually from peak areas (s. Appendix for detailed calculation).

Met and Trp are detected within one peak, Proline can not be found. Asn and Gln are transferred into Asp and Glu due to acid hydrolysis. They can be quantified by dividing the area of the Asn – Asp - peak and Gln - Glu - peak by two. His, Met and Trp get lost through the acid hydrolysis (Lindroth and Mopper, 1997).

External standards were applied to evaluate the yield of the extracted amino acids: Leu for DFAA and Leu - Leu for THAA. To simulate sediment extraction several steps were necessary:

1. Sediment from the wind flat was combusted (4 h, 550 °C) to remove all organic substances.
2. Stock solutions of the standards (10 µmol l^{-1} Leucine; 100 µmol l^{-1} Leucine-Leucine) were prepared in artifical seawater (10 PSU, pH 8).
3. Stock solutions were pipetted onto the organic-free sediment in fireproof crucibles and dried at 100 °C in an oven over night (n = 10).
4. These external standards were analysed as the samples.

2.1.5 Biomass parameters

Chlorophyll *a* was determined to analyse the development of the primary producers. Approximately 1.5 – 2 g fresh sediment (~ 1 cm^3; depth 0.9-1.2 cm) was weighed (Sartorius BP 310S) into clean 15 ml tubes (Sarstedt), mixed (Merck Eurolab) and extracted 2 times with 5 ml of 90 % acetone over night. If the second extraction acounted for more than 15 % of the chlorophyll *a* of the first extraction, a third extraction was performed. Three replicates were extracted always.

The samples were shortly mixed after solvent addition and chlorophyll *a* was extracted at 5 °C over night in the dark. Samples were centrifuged (5.000 rpm, 20 °C, 5 min.; Kendro Laboratory Products Megafuge 1.0R). The extinction of the extract supernatant was measured with a spectralphotometer (Shimadzu UV 2401 PC, slid width 2 nm) in cuvettes (optical glas; Hellma) at 663 nm (extinction maximum of chlorophyll *a*), 630 nm (extinction maximum of chlorophyll c), 750 nm (extinction maximum of bacteriochlorophyll *a*) and 850 nm (turbidity). All wavelengths were corrected by the turbidity.

The pool of chlorophyll *a* can be divided into photosynthetic active chlorophyll *a* and the inactive form, pheophytin. The Mg^{2+}-atom is removed from the active site of the chlorophyll *a* - molecule by acidification and the chlorophyll *a* – molecule is transferred into pheophytin. Especially in sediments, "dead" biomass accumulates and the pheophytin content can amount to 60 – 85 % (Misic and Harriague, 2007).

1 ml of each sample was transferred into 1.5 ml reaction tubes (Eppendorf) and 10 µl of 1 N HCl was added for acidification. After 2 minutes, the sample was measured again and active chlorophyll *a* and pheophytin were calculated (Lorenzen, 1967):

$$\mu g Chla cm^{-3} = \frac{2.43 \cdot (E_{663b} - E_{663a}) \cdot 10^6 \cdot v}{89 \cdot d \cdot V}$$

$$\mu g Phaeo cm^{-3} = \frac{(E_{663b} - 2.43 \cdot (E_{663b} - E_{663a})) \cdot 10^6 \cdot v}{56 \cdot d \cdot V}$$

E_{663b} = extinction before acidification
E_{663a} = extinction after acidification
v = extraction volume (ml)
d = layer thickness of cuvette
V = extracted volume of sediment (cm^3)

Not more than three samples were treated at the same time to keep the reaction time as constant as possible. Chlorophyll *a* was transferred into C-equivalents. It was calculated with the factor 80 µg C µg Chl a^{-1} (Garnier and Maurelatos, 1991).

Bacterial biomass was calculated via cell counts. About 4 – 8 g sediment (~ 1.5 – 4 cm^3, depth 0.9 – 1.2 cm) were weighed into an Erlenmeyer flask. One hundred and fifty ml of Tris-HCL buffer (5 mmol l^{-1}, pH 8.2, 10 PSU) was used to suspend the sediment. The sediment was mechanically suspended by agitation. No ultrasound was used. Substrates for enzymatic activity analyses were suspended in the same ultrasound bath causing head development which might have affected the organisms and their activities. One ml of the supernatant was fixed with 37 % formol (4 % v/v). The cells were counted via epifluorescence microscopy by staining the nucleic acid of the cells with 4', 6-diamidino-2phenyl indole (DAPI) (Porter and Feig, 1980). Therefore, the sample was filtered onto a membrane filter (0.2 µm GTTR; Millipore Isopore ™, 400 mbar max.). The cells were stained with 1 ml of ca. 90 µmol l^{-1} DAPI (1 mg dissolved in 1 ml dimethyl sulfoxide (DMSO), which was diluted in phosphate buffer pH 7.6) for five minutes. The dry filter was mounted with Immersion oil (Olympus Type F) and then counted under the epifluorescence microscope (OLYMPUS BX 51, Filter UV (BP 410), excitation 365 nm, emission 410 – 450 nm). The DAPI signal was good and stable. The stained bacteria faded only after some time. Thus, counting was easy. For each sample, one replicate was counted with about 20 cells in 20 fields marked by an ocular grid (= 400 cells at least). The standard deviation of one sample in six replicate filters was 10 %. Bacterial abundance was transferred into C- equivalents, assuming 10.4 fg C $cell^{-1}$ (Simon and Azam, 1989).

Live-dead staining of unfixed samples was done at three samplings to evaluate the live and potentially active part of the bacterial population (s. Tab. 2.1). The microbial mat was sliced into 3 smaller pieces of 3 mm depth to generate depth profiles. The Live/ Dead® BacLight™ Bacterial

Viability Kit (Invitrogen) consists of two fluorescence dyes testing the membrane integrity of the cells. The dye SYTO® 9 stains the DNA of all cells resulting in a green fluorescence signal under blue excitation. Propidium iodide can only enter the cell when the membrane integrity is lost (= the cell is dead), resulting in a red fluorescence signal under blue excitation. About 2 – 3 g (~1 cm^3) sediment were suspended with 10 – 20 ml of Tris-HCL buffer by agitation. One ml of the supernatant was stained with 3 µl 1:1 mixture of the fluorescence dyes for 5 minutes. The sample was filtered onto a membrane filter (0.2 µm GTTR; Millipore Isopore ™ 400 mbar max.) and then counted under the epifluorescence microscope (Olympus BX51, filter blue (BP 490), excitation 450 – 480 nm, emission 500 – 550 nm). Three replicates with at least 400 cells for either dead or alive bacteria were counted. The fluorescence signal was not as good and stable as the DAPI signal. Green and red fluorescence could be identified well. Unfortunately, red fluorescence faded resp. turned light orange – yellowish and was harder to distinguish from green fluorescence. Green fluorescence faded quite fast. Counting was possible and manageable but it was not possible to take any photos from the filters.

2.1.6 Enzymatic hydrolysis

Model - substrates for different hydrolytic enzymes were used to determine the amount of hydrolysed organic material. Fluorescent model-substrates were applied, because they are used in most studies on aquatic microbes (e.g. Hoppe, 1983). The respective substrate is bound to the fluorophore 4- Methylumbelliferon (MUF) or 7-Amino-4-Methylcoumarin (AMC). As long as the fluorophore is bound to the substrate, the whole molecule is not fluorescing. The fluorophore is cleaved enzymatically from the substrate and the fluorescence increase is detected over a certain time. Standard curves of MUF and AMC were measured every sampling in a geometric dilution series (25 µmol l^{-1} – 0.4 µmol l^{-1}) to convert fluorescence increase into hydrolytic activity.

A linear fluorescence increase is important to guarantee a precise calculation of the enzyme activity. For all detected fluorescence values, linear regressions were calculated.

Two ml of the supernatant (s. bacterial abundance for sample preparation) were transferred into cuvettes (Sarstedt, polystyrol, 10 · 10 · 45 mm). Three replicates were measured for every sample. The model - substrates were suspended within 100 % ethanol and 40 µl of the stock solution/ suspension were added to the cuvettes resulting in final ethanol concentration of 2 %, which did not affect bacterial integrity (Bednorz, 2007). Blanks of ultrapure water with Tris-HCL buffer (three replicates) were measured for chemical hydrolysis of the MUF- and AMC-substrates. Five to six substrate concentrations between 0 – 1000 µmol l^{-1} were used for enzyme kinetics and 1000 µmol l^{-1} as concentration for the depth profiles. Substrate concentrations were transferred into µmol cm^{-3} for better comparability.

All samples were buffered at pH 8.2 and incubated and measured at room temperature.

The fluorescence increase was detected with a HITACHI F 4010 Fluorometer at 365 nm excitation and 451 nm emission (excitation and emission bandpass 1.5 nm; response 2 s, average 2 s). The resulting enzyme activities were expressed either in µmol cm^{-3} h^{-1} or normalized by the bacterial abundance to amol $cell^{-1}$ h^{-1}.

Enzyme kinetics were recorded for every enzyme and were evaluated by the model of Michaelis-Menten: The velocity of the turnover should be only dependent on substrate [S] and product [P] concentration. During the reaction, an intermediate product, the enzyme-substrate-complex [ES], occurs where the substrate is bound to the active site of the enzyme [E] and the enzyme-substrate-complex concentration remains constant.

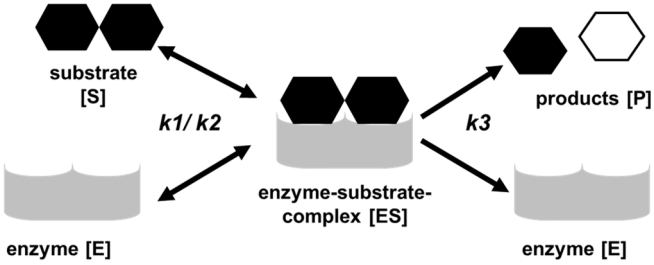


Fig. 2.3: Scheme of the enzymatical cleavage of substrates based on the Michaelis-Menten theory.

After building [ES] with reaction velocity *k1*, ES can decay into [E] and [S] with reaction velocity *k2* or into [E] and [P] with reaction velocity *k3* (Fig.2.3). Usually, *k3* is higher as *k2* so the balance of the reaction is displaced to the product site (IUPAC, 1997).

The kinetics are described by the following equation:

$$v = \frac{(Vmax \cdot [S])}{(Km + [S])}$$

v = resulting mols of the product per time unit

S = substrate concentration

Vmax = potential turnover rate

Km = Michaelis-Menten-constant; substrate concentration with half of the active sites occupied

The hydrolytic activities for one enzyme were plotted against the corresponding substrate concentration. At higher substrate concentrations, the hydrolytic activities do not increase longer. Thus one obtains saturation curves. Michaelis-Menten kinetic parameters were extrapolated with these saturation curves:

Theoretical activities for every substrate concentration had to be guessed following the Michaelis-Menten equation. The differences between these “calculated” and “measured” activities were

squared. They are also normalised by the corresponding "measured" activity for a better comparability between the kinetics. The program Solver in Microsoft Excel generates the Michaelis-Memten parameters by adjusting the Vmax- and Km- values that the summed up deviation squares become minimal.

The obtained Vmax values were used to assess the potential part of hydrolytic enzymes in cycling organic material and to calculate the turnover of proteins and carbohydrates.

Therefore, the results of HPLC analysis for amino acids and literature data were used (s. 4.0.2 and Appendix for details and calculation).

2.1.7 Utilisation of different carbon sources by microbial mat communities

The microbial community was analysed according to their ability to utilise different carbon sources. The Biolog Eco Plate (BIOLOG) contains 31 single carbon sources (3 replicates each + 3 control-wells) in a 96 -well microplate: amino acids, carbohydrates, amines/ amides, carboxylic acids and polymers (classification after Weber and Legge, 2009). The wells are initially colourless. When a carbon source is oxidised due to respiration by the organisms, a tetrazolium dye may be used as an artificial electron acceptor. The reduced tetrazolium dye is purple. Thus, Biolog profiles show carbon source oxidation but not necessarily growth (Konopka et al., 1998)

The microbial mat was sliced into smaller pieces of 3 mm depth until 1 cm of depth in total. About 2 – 3 g (~1 cm^3) sediment were slurried with 10 – 20 ml of Tris-HCL buffer (5 mmol l^{-1}, pH 8.2, 10 PSU) and mechanically suspended by gentle agitation. 50 µl of the supernatant were transferred into the 96 -well microplate and incubated for 1 – 9 days at 20 °C. Every day the plates were analysed. The C-scources oxidised within the first 48 h were regarded as C-scources truly oxidised by the community. C-scources oxidised after 48 h were called addional oxidised C-sources (s. 4.0.3 for details).

2.2 Isolates

2.2.1. Phylogeny and choice of isolates

Heterotrophic bacteria were isolated from the phototrophic layer of the microbial mat within wind flat "Bock" in September 2006 and March 2007 (Heyl, 2007). The nine isolates are stored within bacterial strain collection in the "Angewandte Ökologie und Phykologie" at the University of Rostock. They were sequenced according to their 16SrDNA to assign them phylogenetically (Tab. 2.2; s. Appendix for methodological details)

Strains were chosen for experiments according to their physiology and phylogeny (s. 3.3. for details).

Tab.2.2: Results of the 16SrDNA sequencing of the isolates; BLAST-response with accession number, sequence length (base pair (bp)) and identicalness according to data base NCBI.

strain	sequence length (bp)	accession no.	identicalness (%)	BLAST response
9.06 B1	1006	FJ546062.1	99	*Pseudoalteromonas sp.*
9.06 B2	1038	AB186498.1	99	*Vibrio sp.*
9.06 B3	875	EF685673.1	99	*Marinobacter sp.*
9.06 B4	904	AM913917.1	100	*Pseudoalteromonas sp.*
9.06 B5	902	EF685673.1	99	*Marinobacter sp.*
3.07 B1	929	GQ903402.1	99	*Bacillus baekryungensis*
3.07 B2	1377	AM421975.1	99	*Pseudomonas sp.*
3.07 B3	365	CP001252.1	100	*Shewanella baltica*
3.07 B4	929	GQ327990.1	99	*Shewanella sp.*

2.2.2 Experimental strategy

The ability of bacteria to cope with changing quality of substrates was one focus in this study (Tab.2.3). Two approaches dealt with the utilization of substrates ranging from labile low molecular weight compounds like amino acids and monosaccharids to refractory high molecular weight compounds like polysaccharids and polysorbats (**1**). Strains were screened for their constitutive glycolytic enzymes (**2**).

Growth status of the culture and C-source influence the enzyme activities. Hence, the influence of C-sources on hydrolytic activities was analysed. In general, bacterial abundance was examined at several points during incubation to evaluate the growth status of the cultures.

These general questions were analysed in three approaches: The influence of different C-sources on constitutive β-glucosidase (**A**), the inducibility of β-glucosidase by different C-sources (**B**) and the influence of different C-sources of α-glucosidase (constitutive in both bacterial strains chosen) and β-glucosidase (constitutive in one bacterial strain, inducibile in the other) for 10 days of growth (**C**). The incubation was 10 days long to guarantee that all cultures reached stationary growth phase. Hydrolytic activities were measured at different growth statuses of the cultures. Experimental duration became longer during the study, starting from one day and ending with ten. Bacteria grew different in every experiment and sometimes stationary phase was not reached.

Tab.2.3: Overview of the performed experiments with isolates. chosen strains, duration of experiments and examined hydrolytic activities.

experiment	isolates	conditions	examined enzymes
(1) - utilization of substrates (BIOLOG)	9.06 B1 9.06 B4 3.07 B3 3.07 B4	- 24 h	-
(2) - constitutive enzymes	9.06 B1 9.06 B4 3.07 B3 3.07 B4	24 h	α-glucosidase β-glucosidase cellobiodase N-Acetylglucosaminidase
(A) – influence of C-sources on growth and constitutive enzymes	3.07 B4	1 – 3 d	β-glucosidase
(B) - induction of enzymes with different C-sources and influence on growth	9.06 B4	1 – 3 d	β-glucosidase
(C) – influence of C-sources on constitutive and inducible enzymes and on growth	9.06 B4 3.07 B4	2 - 10 d	α-glucosidase β-glucosidas

2.2.3. Substrate profiling

Biolog GN2-microplates (BIOLOG) were used to create a metabolic fingerprint for the bacterial isolates of the wind flat microbial mat. The GN2-microplate contains 95 single carbon sources and one control-well (H_2O) and is based upon redox chemistry combined with a purple dye as signal for carbon source respiration (for details s. 2.1.7; C-scource classification after Garland and Mills, 1991). The organisms were grown on peptone and yeast-extract containing liquid medium (Seawater yeast peptone medium (SYP) 10 PSU, pH 8; (s. Appendix for details)) for 24 h. Cells were harvested by centrifugation (Kendro Laboratory Products Megafuge 1.0R; 5000 rpm, 5 min, 10 °C). The pellet was resuspended with 10 ml Tris-HCL (10 PSU; pH 8.2). Plates were incubated with 100 µl per well for 1 h at 20 °C. Only wells showing a dark purple staining were analysed as positive because the control wells stained also light purple.

2.2.4. Hydrolytic activity depending on growth and carbon source

All experiments were carried out by utilising 24 h preparatory cultures on SYP medium. Two media were used to assess the substrate influence: minimal medium (s. appendix for details) with one carbon source (0.5 % mass) and SYP medium with an extra carbon source (0.5 %). These “general” SYP medium was applied to guarantee an omnipotent status of the cells, even when the influence

of different C-sources on enzyme activity was analysed. Furthermore, not all bacterial isolates could grow on minimalmedium. Carbon sources were cellobiose (Sigma) as possible inducer of β-glucosidase, soluble starch (Sigma) as possible inducer of α-glucosidase and D-Glucose (Sigma) as possible inhibitor of α- and β-glucosidase. As the polymere starch has no "real" molecular weight, but consists only of glucose, the same amount like glucose was used. Bacteria were cultured within Erlenmeyer flasks on a shaker (150 rpm) at 20 °C.

2.2.4.1 Growth rates

Bacterial abundance and hence growth status of the culture was determined at 4 – 12 points during incubation. 450 µl culture were fixed with 37 % formol (9 % v/v) and cells were counted in a haemocytometer (Bürker, Labor Optik) under a light microscope (Olympus BH2). At least 400 cells were counted. Standard deviation was determined with 13 % by counting three parallels of 42 samples representing growth for 28 h.

Bacterial growth in batch cultures can be separated into different phases resp. statuses (Fig. 2.4). Bacteria need some time to adopt themselves to the medium in the lag-phase. Afterwards exponential growth starts. Bacteria double proportionally to time. The slope of this line is the growth rate of the culture. This model is called exponential growth. The exponential phase is not infinite. Bacterial culture reaches stationary phase in which cell-division (new cells) and cell-lysis (cell dead) are more or less on the same level. Stationary phase begins when some factors necessary for growth (e.g. nutrients) become limited. The culture is characterised by its capacity, the maximal cell number reached under the respective conditions. This model is called logistical growth.

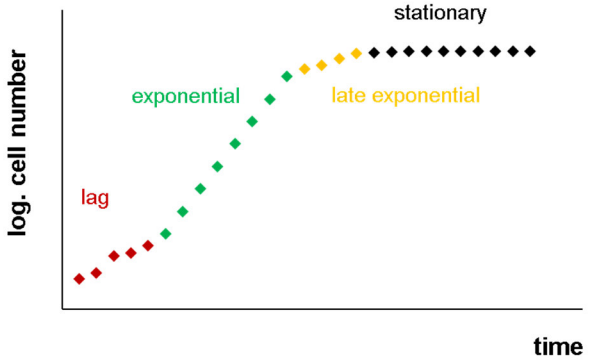


Fig. 2.4: Different bacterial growth phases: lag-phase (red), exponential phase (green), late exponential phase (yellow) and stationary phase (black). Cell numbers plotted logarithmic against time.

Both models of growth can be described with the following formulae:

Exponential growth:

$$N(t) = N(0) \cdot e^{\mu \cdot (t - t1)}$$

N (0) = cell number at beginning of growth (cells · 10^6 ml^{-1})
N (t) = cell number after time t (cells · 10^6 ml^{-1})
μ = growth rate (d^{-1})
t = time of growth (d)

Logistical growth:

$$N(t) = \frac{K \cdot N(0) \cdot e^{\mu \cdot t}}{K + N(0) \cdot (e^{\mu \cdot t}) - 1}$$

N (0) = cell number at beginning of growth (cells · 10^6 ml^{-1})
N (t) = cell number after time t (cells · 10^6 ml^{-1})
μ = growth rate (d^{-1})
t = time of growth (d)
K = capacity (cells · 10^6 ml^{-1})

Growth rates were calculated with both models for all experiments.
Cell numbers were plotted against time. Theoretical cell numbers were calculated taking cell numbers at t(0) as base and assuming a growth rate. The real growth rates μ and capacities K were assessed with the program Solver in Microsoft Excel like done for the Michaelis-Menten kinetics (s. 2.1.6).
Additionally, the doubling time was calculated:

Doubling time (dt)

$$dt(h) = \frac{\ln(2)}{\mu}$$

2.2.4.2 Protein content

The cellular protein content was analysed analogue to bacterial abundance (**A**, **B**). Cells were harvested by centrifugation (6.000 rpm, 10 min, 20 °C), suspended with 1 ml Tris-HCl (5 mmol l^{-1}, 10 PSU, pH 8) and stored at -20 °C. After centrifugation (13.000 rpm, 5 min), the pellet was suspended with 0.5 ml Tris-Hcl. Cells were disrupted by ultrasound (30 s, 40 % intensity, MS 64, Bandelin Sonopuls HD). This was repeated three times under cooling and addition of bead-beat-

perls (ø 100 µm). Samples were centrifugated (13.000 rpm, 10 and 30 min) twice to clean the proteins from cell residues (Bradford, 1976, mod.). Proteins were stored at -20 °C . Proteins were analysed fluorometically using Quant_iT^{TM} Protein Assay Kit (Invitrogen) for Qubit fluorometer. Every sample was measured in three replicates and bovine serum albumin (BSA) was used as a standard (0, 200, 400 ng BSA µl^{-1}). All protein concentrations are expressed in BSA-equivalents.

2.2.4.3 Hydrolytic enzyme activity

10 ml culture was centrifugated (6.000 rpm, 5 min, 20 °C) to harvest the cells. Cells were washed twice with Tris-HCl (5.000 rpm, 5 min, 20 °C) in order to rinse out all substrates influencing the enzyme activity. Additionally, 1 ml of the washed culture was fixed with 37 % formol (4 % v/v) to get the actual bacterial abundance generating hydrolytic activity. Cells were counted in a haemocytometer (Bürker, Labor Optik) under light microscope (Olympus BH2). At least 400 cells were counted.

MUF-substrates were applied (for theoretical and methodical background s. 2.1.6). Concentration of the according substrate was 750 – 800 µmol l^{-1}. Samples were measured three times within 4 – 6 h (**A**). Hydrolytic activity was measured every 24 h (**B**) or 7 times during 10 days experiment (**C**). All hydrolytic activities were normalized by cell numbers.

2.3 Statistics

All statistics were performed with program IBM SPSS Statistics (Version 20).

2.3.1 Correlations

Correlation analyses were performed to test if two parameters are potentially influenced by each other or if they are connected with each other.

Spearman-Rho correlation was applied. It was only applied if the asymptotic significance was < 0.05. The Spearman-Rho coefficients generates absolute values between 0 and 1. The higher the absolute value the higher is the potential connection of the parameters. Positive values mean a positive correlation, negative ones a negative one.

Obtained coefficients were classified as followed: < 0.2 no, 0.2 – 0.5 weak, 0.5 – 0.7 mediocre, 0.7 – 0.9 strong and > 0.9 very strong correlation.

2.3.2 Significant differences between samples

Most environmental samples do not feature a Gaussian distribution. Hence, non-parametric tests have to be performed. Significant differences in a data set with more than two groups can be found with the non-parametric Kruskal-Wallis test ($p < 0.05$). It just shows that groups within the data set are significantly different from each other. It does not identify the groups which are significantly different from each other. The groups have to be tested one by one as Post-Hoc tests are not possible in non-parametric data sets. This was done with non-parametric Mann-Whitney test, which compares two groups with each other. All possible combinations have to be tested one by one as the data sets consisted of more than two groups. This means an increase of the alpha mistake. Hence, a correction has to be made for the asymptotic significance p. This was done with the Bonferroni correction k:

$$k = \frac{p}{n}$$

p = asymptotic significance

n = number of Mann-Whitney tests

Hence, asymptotic significance p becomes smaller. In a data set of 6 groups, 15 combinations are possible and asymptotic significance p becomes 0.0033. In a data set of 9 groups, 36 combinations are possible and asymptotic significance p becomes 0.0014. Hence, not all groups were compared with each other, but they were chosen as followed:

Only groups following behind each other in time were compared (May and June, June and July, July and August..../ day 1 and 2, day 2 and 3) to identify the moment when significant changes occurred. The significances $p < 0.05$ are also given as the Bonferroni-correction is a very conservative correction, being aware of the incorrectness in terms of statistical accuracy.

3. Results

3.1 Heterogeneity within the wind flat microbial mat

3.1.1 Spatial heterogeneity

The spatial heterogeneity within the microbial mat was analysed for May 2009. The biomass parameters chlorophyll *a*, pheophytin and bacterial abundances are reported for increasing distances (2 – 100 cm) in the four cardinal directions from one central point. The "heterogeneity map" (Fig. 3.1) shows the uneven distribution of bacteria and primary producers. The biomasses accumulated at different spots, but these hot spots of bacteria and primary producers did not necessarily overlap. The chlorophyll *a* amount ranged between 12 – 37 µg cm^{-3} and varied by 1 – 47 % around the median (23 µg cm^{-3}). The pheophytin amount with 5 – 13 µg cm^{-3} accounted for 13 – 40 % of the total pigment amount.

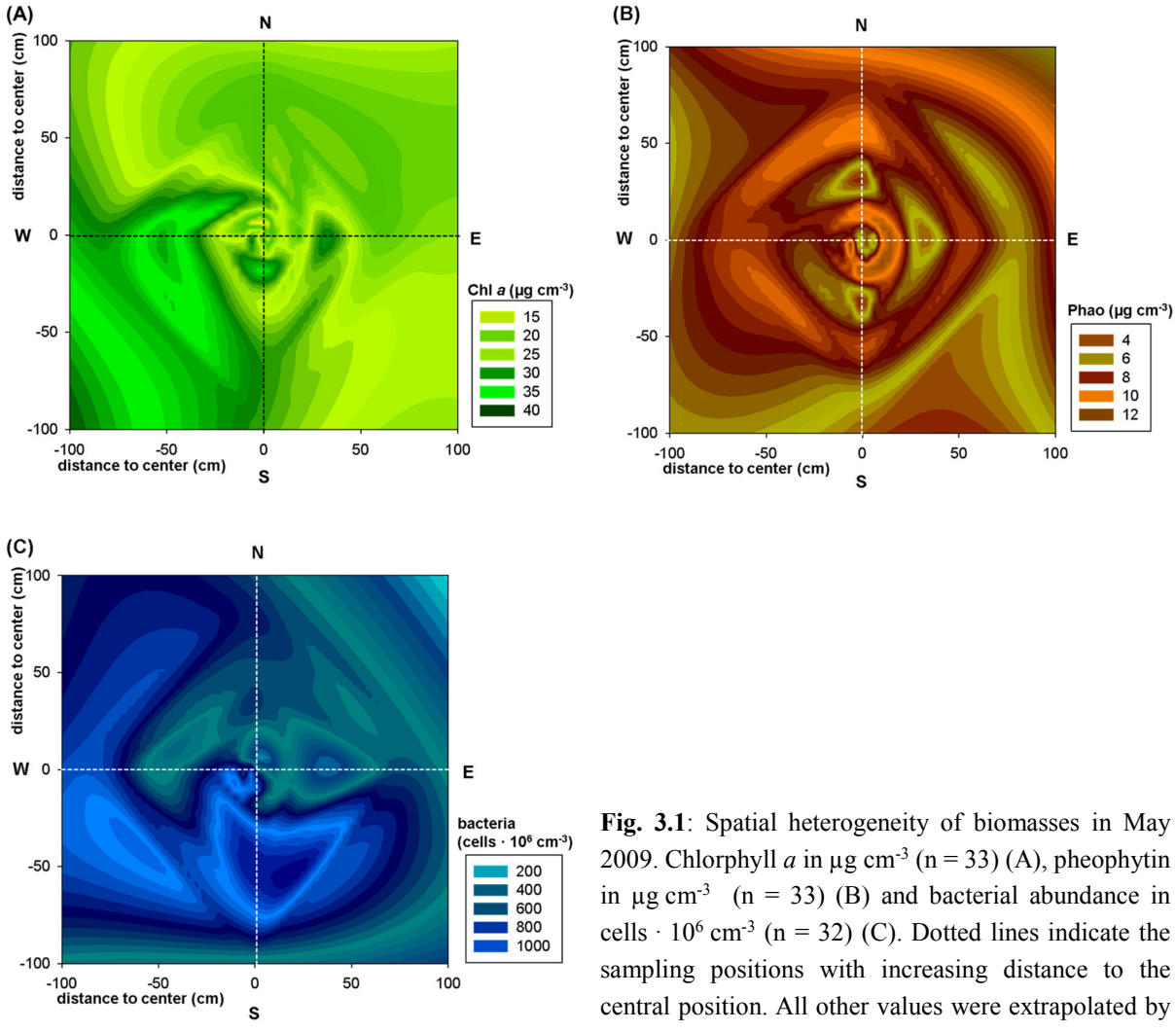


Fig. 3.1: Spatial heterogeneity of biomasses in May 2009. Chlorphyll *a* in µg cm^{-3} (n = 33) (A), pheophytin in µg cm^{-3} (n = 33) (B) and bacterial abundance in cells · 10^6 cm^{-3} (n = 32) (C). Dotted lines indicate the sampling positions with increasing distance to the central position. All other values were extrapolated by the program Sigma plot.

Bacteria varied between 250 – 1,110 cells · 10^6 cm^{-3}. The bacterial abundances were more widely and heterogenically distributed than the primary producers. The median was 630 cells · 10^6 cm^{-3} and the abundances ranged between 0.5 – 100 % around it.

The Spearman-Rho correlation coefficients between chlorophyll *a* and pheophytin (0.087), chlorophyll *a* and bacteria (0.194) and pheophytin and bacteria (-0.335) showed no or only a weak relation between these parameters.

3.1.2 Depth profiles

The depth profiles for chlorophyll *a* and bacteria were investigated in May, August and November 2009. The upper layer (0 – 3 mm) was always green. The lower layer was yellow-green in colour (3 – 7 mm) and the lowest layer showed sometimes little black spots (7 – 10 mm). The black layer, indicating FeS, was mostly found deeper than 10 mm. It varied in width and intensity (Fig. 3.2). Only in July 2009, it was directly located below the green layer (Fig. 3.2 E).

The profiles showed a clear biomass accumulation within the upper layer of the microbial mat throughout the year. The active chlorophyll *a* ranged between 50 – 65 µg chl *a* cm^{-3} in the upper microbial mat layer (0 – 3 mm) and decreased by 75 – 55 % in the lower layers (3 – 7, 7 – 10 mm) (Fig. 3.3). The live bacteria amounted for 530 – 850 cells · 10^6 cm^{-3} in the upper microbial mat layer and decreased by 50 – 80 % in the lower layers (Fig. 3.4).

The lower layers (3 – 7, 7 – 10 mm) did not differ very much in their biomasses. Chlorophyll *a* and bacterial abundance decreased further in the lowest layer only in one month each. The chlorophyll *a* amount was 30 % in the 7 – 10 mm layer lower than in the 3 – 7 mm layer in August 2009 (Fig. 3.3 B). The bacterial abundance in the 7 – 10 mm layer was 50 % lower than in the 3 – 7 mm layer in May 2009 (Fig. 3.4 A).

The layers did not differ significantly in the percental distribution of active chlorophyll *a* and live and potential active bacteria ($p > 0.05$ for active chlorophyll *a* and live bacteria; Kruskal-Wallis). 70 – 90 % of the primary producers and the heterotrophic bacteria were alive and potential active. The seasonal variety within the singles layers was not significantly different for active chlorophyll *a* and live bacterial abundance ($p > 0.05$; Kruskal-Wallis).

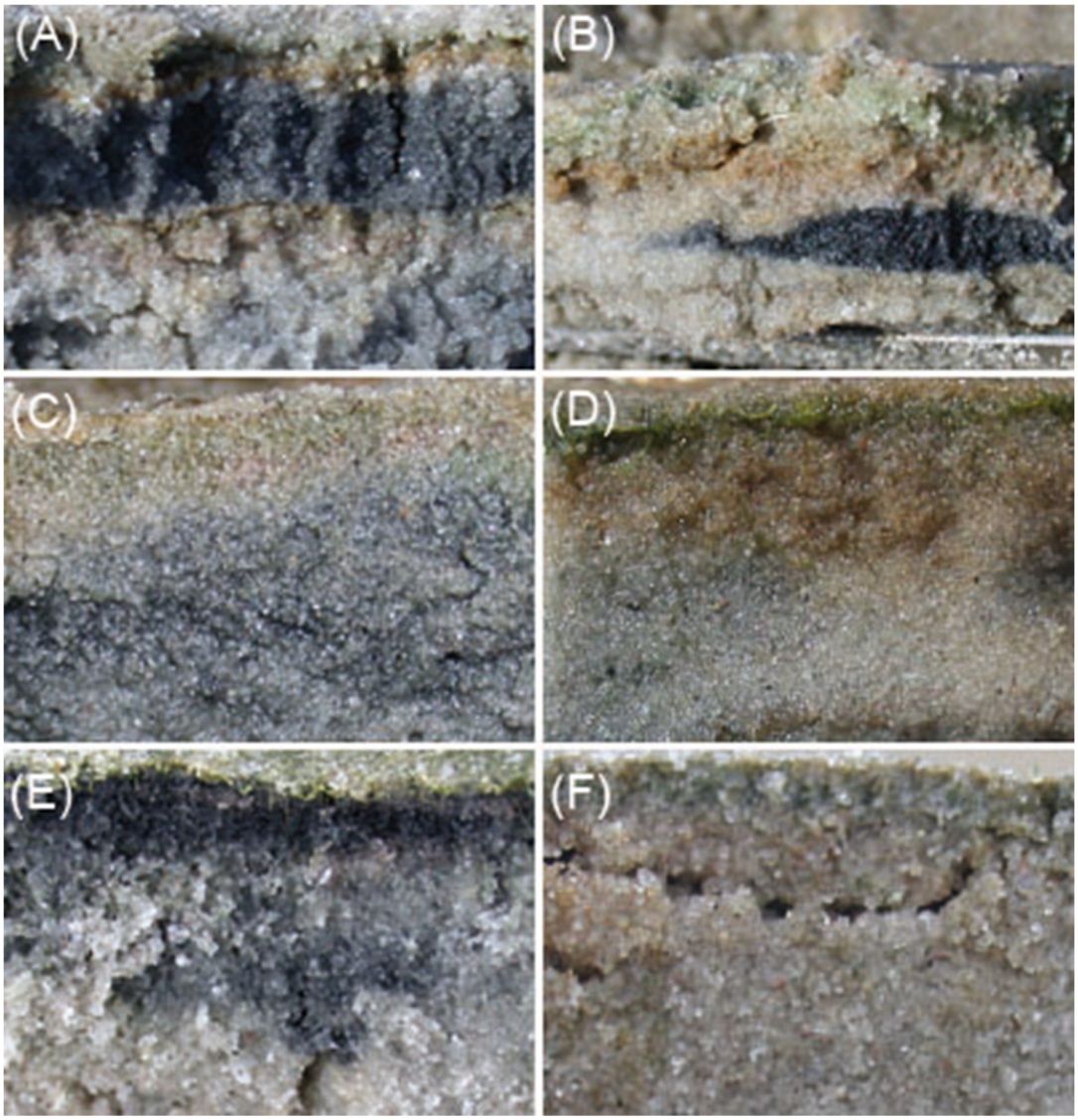
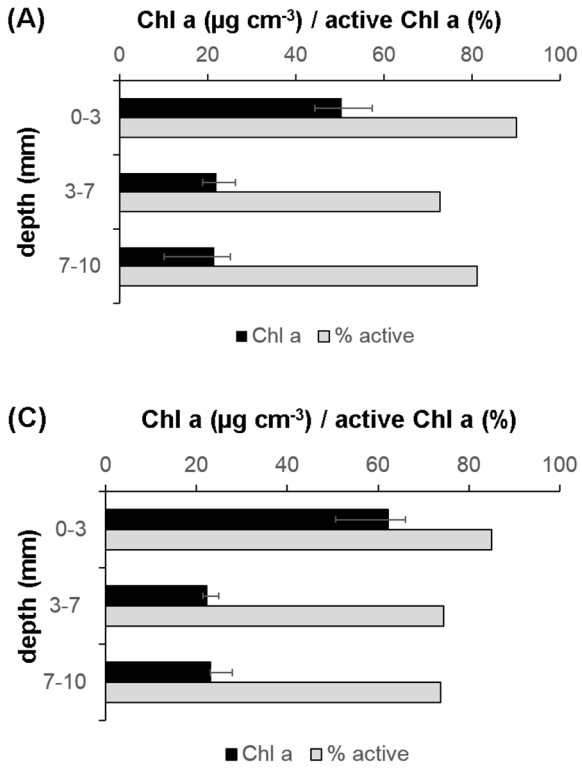


Fig. 3.2: Microbial mat depth profiles in wind flat “Bock” in August 2008 (A), September 2008 (B), November 2008 (C), June 2009 (D), July 2009 (E) and August 2009 (F).



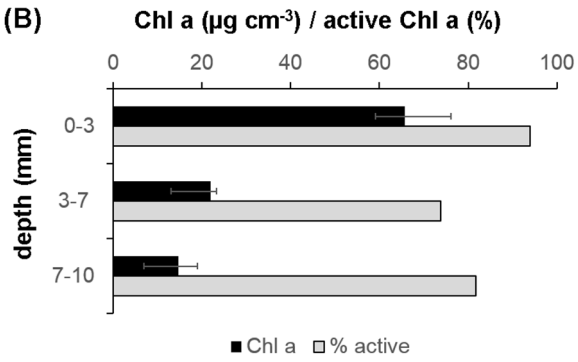
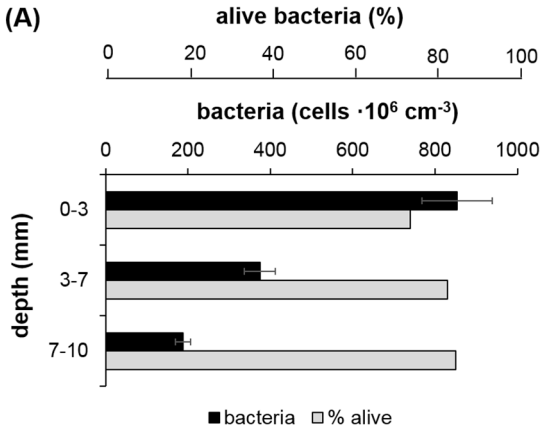
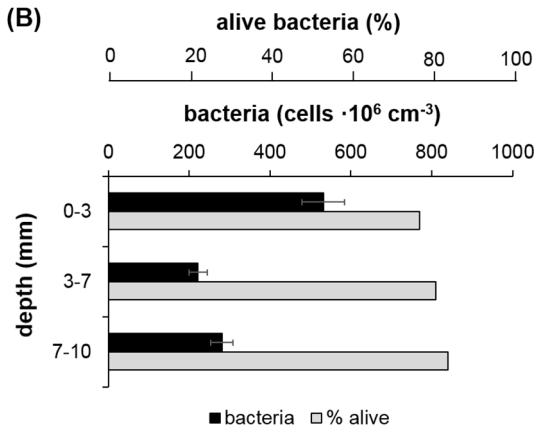


Fig. 3.3: Depth profile of active chlorophyll *a* in µg cm^{-3} (black bars) and % (light bars) within the microbial mat in depth of 0 – 3, 3 – 7, 7 – 10 mm for May (A), August (B) and November 2009 (C) (n = 3). Median with minima and maxima.





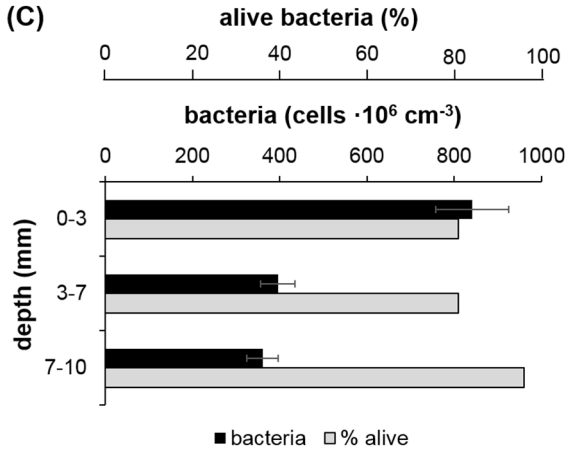


Fig. 3.4: Depth profile of alive bacteria in cells · 10^6 cm^{-3} (black bars) and % (light bars) within the microbial mat in depth of 0 – 3, 3 – 7, 7 – 10 mm for May (A), August (B) and November 2009 (C) (n = 3). Mean and standard deviation.

3.1.3 Seasonal heterogeneity

Meteorological data during the sampling period were obtained from the weather station of the Federal Environment Agency at Zingst, located ~11 km west of the wind flat. Temperature was between 0.4 – 5 °C in winter and between 14 - 20 °C in summer (Fig. 3.5 A). The area was covered by a thin ice shield and the microbial mat was frozen on sampling dates in January and February 2009. The area was additionally covered by floating crushed ice in February.

Global radiation was with 230 W m^{-2} highest in May and June and varied between 190 – 220 W m^{-2} in August and September (Fig. 3.5 B). The annual mean global radiation was 120 W m^{-2}. Precipitation ranged between 40 – 65 mm (Fig. 3.5 C). It was lowest in April (5 mm) and highest in November (85 mm).

The prevailing winds came from southern directions (ESE to SSW), supporting a non-flooded wind flat during the whole year 2009. The wind flat was flooded on three sampling dates: February, June and October. The microbial mat itself was not flooded in February. The water was about 30 cm depth directly over the microbial mat in June and about 60 cm in October.

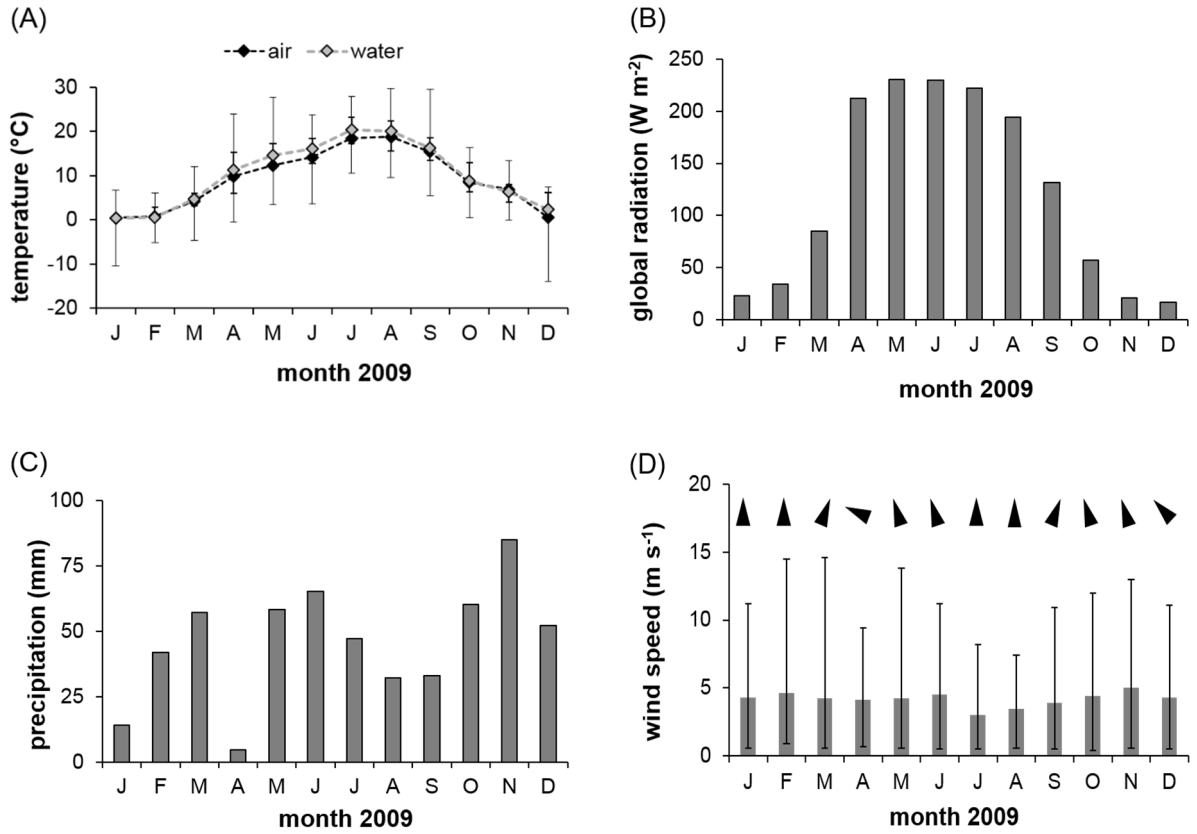


Fig. 3.5: Weather conditions during observation in 2009 at meteorological station Zingst located approximately 11 km west of sampling site: Mean temperature in °C of air and water with minima and maxima (A), mean global radiation in W m^{-2} (B), sum of precipitation in mm (C) and mean wind speed in m s^{-1} (bars) with minima and maxima and direction (arrows) (D).

The average wind speed was around 3 – 5 m s^{-1} during the whole sampling period. In peaks the wind speed reached up to 15 m s^{-1}, especially in February and March when usually spring gales occur (Fig. 3.5 D).

Water- and organic content as general microbial mat characteristics were recorded over the whole sampling period. The highest water contents with > 20 % dry mass (DM) were found during ice coverage- and flooding- period. The water content was slightly lower with 17 – 19 % DM the rest of the year (Fig. 3.6). The organic content ranged between 0.6 – 4.5 % DM. The organic content was lowest in March (0.6 % DM). It fluctuated around 1 – 1.5 % the rest of the year.

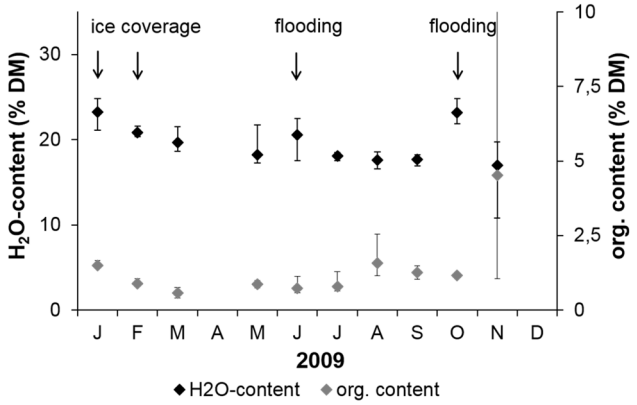


Fig. 3.6: H_2O content in % dry mass (DM) and organic content in % dry mass (DM) of the microbial mat in the wind flat "Bock" during 2009. Median with minima and maxima. January – July n = 3; August – November n = 10; April and December: no data.

No purple layer of phototrophic purple sulfur bacteria was visible within the wind flat microbial mat by naked eye. However, the photometric extinction at 750 nm (absorption maximum of bacteriochlorophyll *a* in acetone) showed the presence of this for microbial mats typical group throughout the year (Fig. 3.7). The photometric extinction at 750 nm ranged between 0.02 – 0.04, whereas the photometric extinction at this wavelength in mat-free sediment was always around 0 (data not shown). The photometric extinction at 630 nm, absorbence maximum of chlorophyll *c* (Bacillariophyceae) in acetone was 2 – 5 times higher than the one at 750 nm. The relative photometric extinction at 663 nm (absorbence maximum of chlorophyll *a* in acetone) was 10 – 20 times higher than the one at 750 nm.

The microbial mat chlorophyll *a* – amount as general parameter for the primary producers fluctuated between 19 – 44 µg cm^{-3} over the year 2009 (Fig. 3.8 A). Considering the high chlorophyll *a* amount in February (44 µg cm^{-3}) as an outlier, the primary producer population rose constantly until July (35 µg chl *a* cm^{-3}) and decreased by 2 – 17 % until the end of 2009.

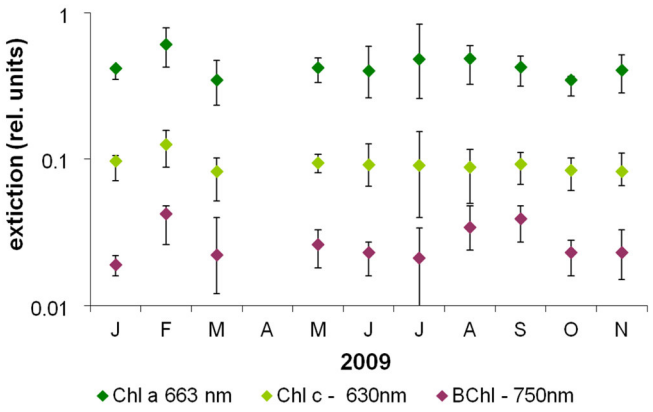


Fig. 3.7: Photometric extinction in logarithmic relative units at the first out of three pigment extractions for chlorophyll *a* (Chl *a*) at 663 nm (dark green), chlorophyll *c* (Chl *c*) at 630 nm (light green) and bacterial chlorophyll (Bchl) at 750 nm (purple) during 2009. Extinctions are corrected by turbidity blank at 850 nm. Median with minima and maxima. January – July n = 3; August – November n = 10.

The inactive chlorophyll *a* form, pheophytin, regarded as "dead" primary producer biomass accounted with 3 – 12 µg cm^{-3} for 6 – 30 % of the total pigment amount (Fig. 3.8 B). The lowest pheophytin amounts were found during summer in July – August (3 – 4 µg cm^{-3} = 8 – 10 %), when the chlorophyll *a* amount was highest (assuming the low pheophytin amount in February (3 µg cm^{-3}) to be an outlier). During the rest of the year the pheophytin amount made up between 20 – 30 % of the total pigment amount.

The bacterial abundance varied between 50 – 450 cells · 10^6 cm^{-3} over the year (Fig. 3.8 C). The bacteria population was lowest during the ice coverage in January and February 2009. It rose up to 450 · 10^6 cells cm^{-3} (June), then declined to 200 · 10^6 cells cm^{-3} and fluctuated around 300 · 10^6 cells cm^{-3} for the rest of the year.

Statistical analysis were applied to test between which months the biomasses significantly changed. The non-parametric Mann-Whitney test with a significance $p < 0.006$ (Bonferroni-correction for k = 9) revealed for chlorophyll *a* a significant difference between January to February, for pheophytin between March to May and August to September and none for the bacterial abundances (Abb. 3.8).

Biomasses were converted into organic C-equivalents (s. Material and methods for details). Furthermore, organic content was converted into organic carbon, assuming that 50 % of the organic mass are carbon. Total organic carbon ranged between 0.7 – 4.3 mmol cm^{-3}. It was highest in November and lowest in March 2009 (Fig. 3.9 A). The biomasses of primary producers and bacteria contributed to 6 – 37 % to total organic carbon.

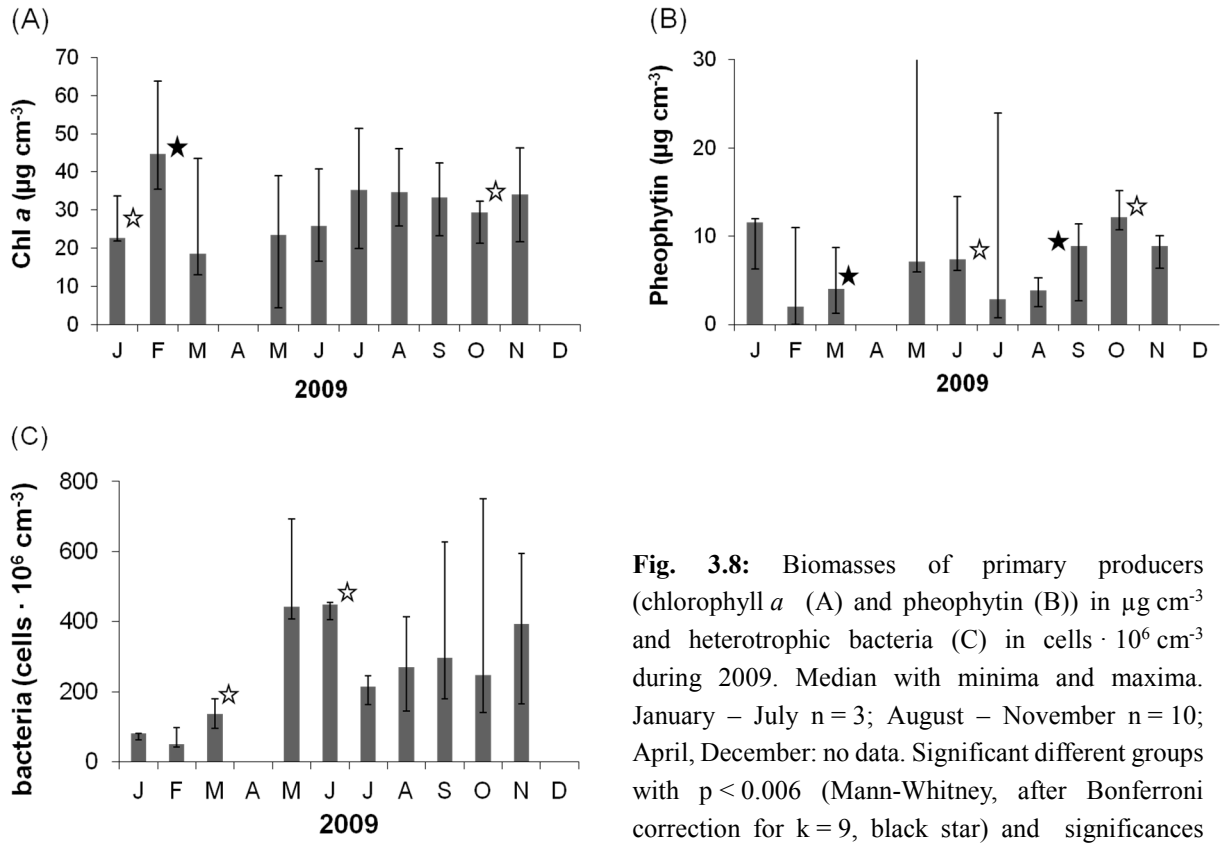


Fig. 3.8: Biomasses of primary producers (chlorophyll *a* (A) and pheophytin (B)) in µg cm^{-3} and heterotrophic bacteria (C) in cells · 10^6 cm^{-3} during 2009. Median with minima and maxima. January – July n = 3; August – November n = 10; April, December: no data. Significant different groups with $p < 0.006$ (Mann-Whitney, after Bonferroni correction for k = 9, black star) and significances $p < 0.05$ (Mann-Whitney, white star).

The organic C of the primary producers made up 80 – 90 % of organic carbon bound in biomass. In January and February, the primary producers even represented 97 – 99 % of the organic carbon bound in biomass.

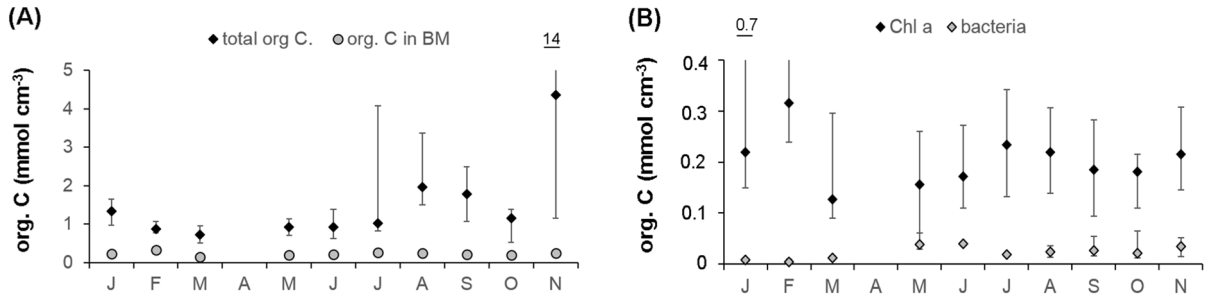


Fig. 3.9: Organic carbon in microbial mat in 2009. Organic content converted into organic carbon in mmol C cm^{-3} (A) and chlorophyll *a* and bacterial abundance converted into C-equivalents in mmol C cm^{-3} (B). Median, minimal and maximal value.

3.2 Organic material, hydrolytic enzymes and their kinetics in the microbial mat

3.2.1 Amino acids and peptides/ proteins in microbial mat

The amino acid pool in the microbial mat was analysed with HPLC in October 2009 to evaluate the potentially available substrates for proteolytic activities. The amino acid pool is divided into the dissolved free amino acids (DFAA), the dissolved combined amino acids (DCAA) and the particular amino acids (PAA). They all sum up to the total hydrolysabla amino acids (THAA). Only DFAA and THAA was identified with the used methods in this study. DCAA resp. PAA were calculated by subtracting DFAA from THAA (s. Chap. 2.1.4). THAA in the wind flat microbial mat accounted for 0.2 – 0.4 mmol C cm^{-3} in October 2009. They made up between 20 – 45 % of the total organic carbon, evaluated via organic content. DFAA contributed with 7 – 35 % to THAA. The majority of amino acids in the microbial mat belonged to DCAA resp. PAA (65 – 93 %) (Fig.3.10).

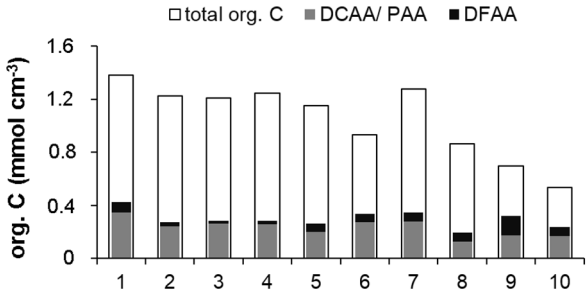


Fig. 3.10: DFAA (black box) and DCAA/ PAA (grey box) amount of total org. C (white box) in mmol C cm^{-3} in 10 samples from microbial mat in October 2009. Only median is shown for reason of clarity and comprehensibility (n = 3).

Not all amino acids could be quantified via HPLC due to methodological reasons or they were not present within the wind flat microbial mat.

Pro could not be identified at all. Tau and Val were not present within the samples. Met and Trp were detected within one peak in the HPLC- chromatogram. Unfortunately, Arg could not be identified in DFAA as it was eluted together with the OPA within one peak (Fig. 3.11 A). Asn and Gln were transferred into Asp and Glu due to the acid hydrolysis for THAA determination. His, Met and Trp got lost through the acid hydrolysis in the THAA (Fig. 3.11 B).

The yield of the external standard Leu was 46 – 77 % (median 62 %) for DFAA. The yield of the external standard Leu-Leu was lower with a yield of 17 – 26 % (median 19 %) for THAA. The DFAA were more unevenly distributed than the THAA. In one sample with three replicates the standard deviation was 50 % in average but sometimes it accounted for more than 100 %.

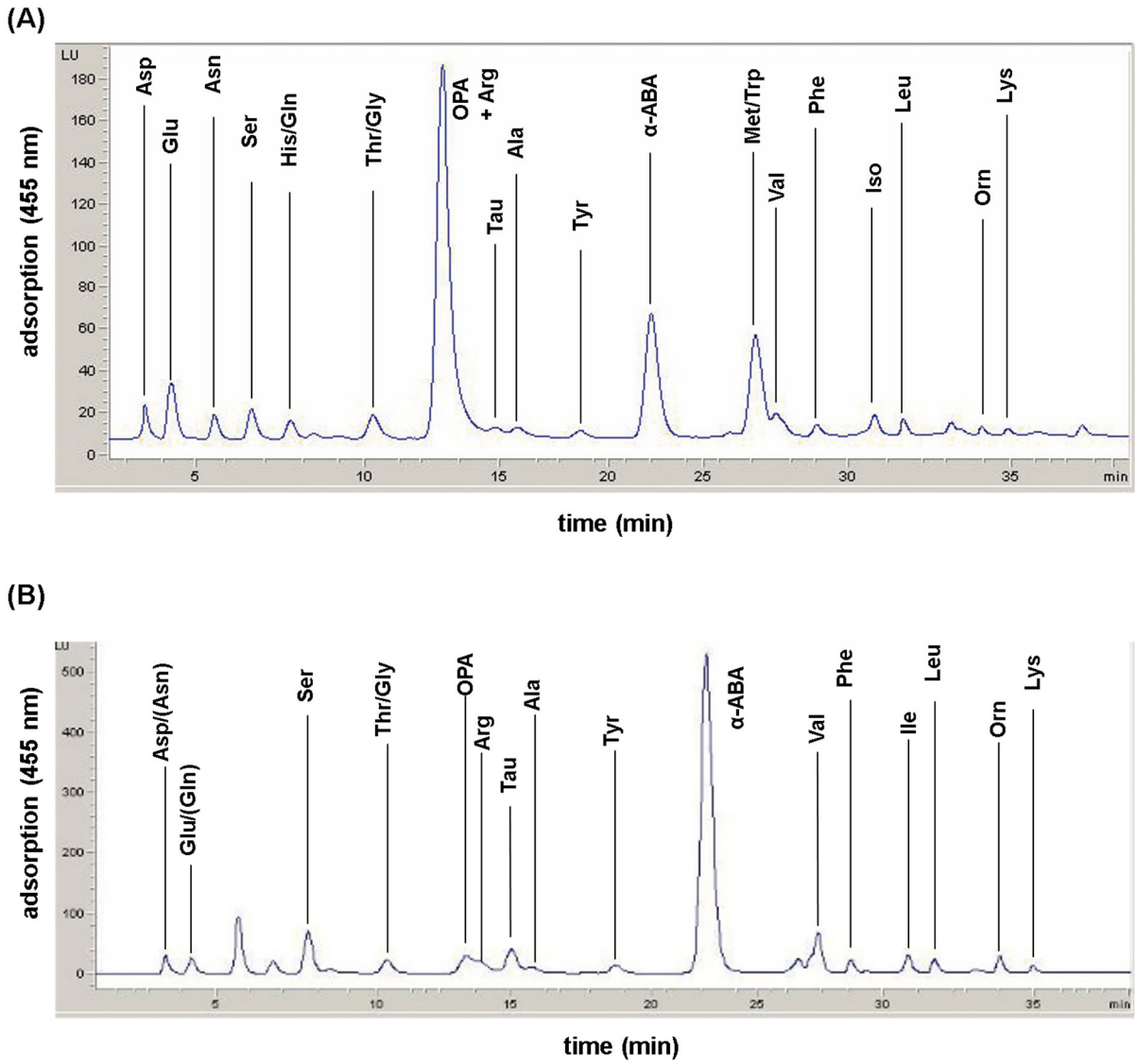


Fig. 3.11: HPLC chromatgrams of amino acid standard for DFAA (A)and THAA (B). Adsorption of amino acid at 455 nm at its characteristic retention time.

The standard deviation for the THAA samples averaged for 20 % and was few times up to 60 – 80 % (Fig. 3.12).

The amino acid composition was more heterogeneously distributed within DFAA than within THAA. The amino acid amount of Ala, Leu, Phe and Arg, whose aminopeptidase activities were measured, could not be identified as being very high compared to the others (Fig. 3.12, Tab. 3.1). Comparing only these four amino acids, all four amino acids were found with more or less the same amount within the DFAA and DCAA/ PAA (except Arg for DFAA) (Tab. 3.2).

Tab. 3.1. Percentage distribution of amino acids in DFAA and THAA in the wind flat microbial mat in October 2009. Minima and maxima, n.m.f.= not found due to methodological reasons; n.f. = not found in sample, but found in standard

Amino acid	DFAA (%)	THAA (%)	Amino acid	DFAA (%)	THAA (%)
Ala	2 – 12	2 – 5	Lys	6 – 18	5 – 21
Arg	n.m.f.	2 – 15	Met	2 – 7	n.m.f.
Asn	0.3 – 1	5 – 10	Orn	1 – 19	0.2 – 1
Asp	2 – 8	5 – 10	Phe	2 – 16	6 – 11
Glu	4 – 10	6 – 12	Ser	1 – 4	4 – 16
Gln	0 – 0.4	6 – 12	Tau	n.f.	n.f.
Gly	1 – 4	2 – 3	Thr	0 – 0.3	3 – 5
His	0 – 0.9	n.m.f.	Trp	11 – 31	n.m.f
Iso	8 – 18	4 – 7	Tyr	0 – 10	5 – 9
Leu	4 – 17	7 – 13	Val	n.f.	n.f.

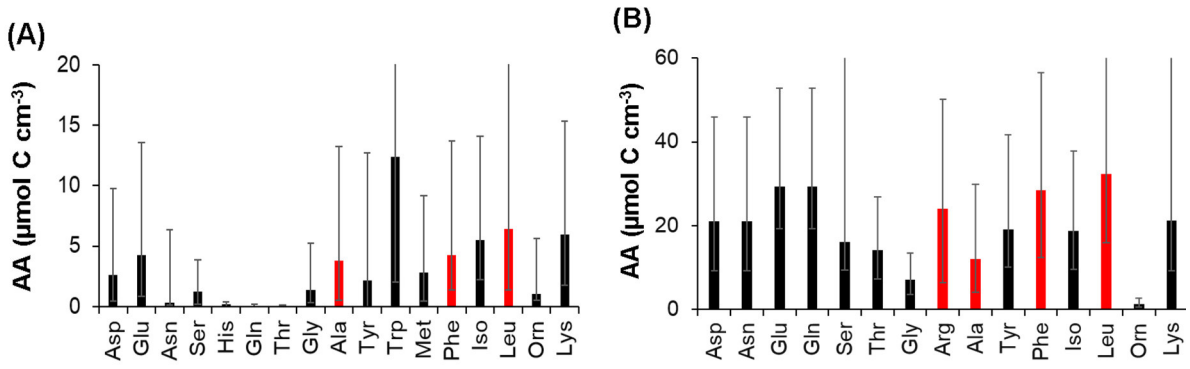


Fig. 3.12: Amino acids in µmol C cm^{-3} found in DFAA (A) and THAA (B) with HPLC-analysis (n = 10). Median and minima, maxima. Amino acids, whose corresponding aminopeptidase activitiy was measured, are marked red.

As mentioned before, Arg could only be found in THAA. Thus, the total Arg amount was regarded to be DCAA resp PAA. Interestingly, the degraded form of Arg, namely Orn, was found with the lowest amount in THAA. It accounted only for 0.2 – 1 % of THAA, whereas in DFAA it accounted for 1 – 19 % (Tab. 3.1).

Tab. 3.2: Amount of the amino acids whose corresponding aminopeptidase activity was measured in THAA, DFAA and DCAA/ PAA in µmol C cm^{-3} (n = 10).

Amino acid	THAA (µmol C cm^{-3})	DFAA (µmol C cm^{-3})	DCAA/ PAA (µmol C cm^{-3})
Ala	7 – 24 (13)	0.8 – 8 (4)	3 – 19 (7)
Leu	11 – 27 (17)	0.8 – 12 (3)	5 – 24 (15)
Phe	6 -15 (10)	0.5 – 4 (2)	4 – 14 (8)
Arg	8 – 20 (12)	0	8 – 20 (12)

3.2.2 Hydrolytic acticvities within microbial mat

Hydrolytic enzyme activities were analysed to assess the importance of hydrolytic enzymes for the organic material turnover within the wind flat microbial mat.

A linear fluorescence increase of the applied MUF- resp. AMC- substrate is important to guarantee a precise calculation of the enzyme activity. Aminopeptidases were measured for up to 4 hours, glycosidases for up to 22 hours (Fig. 3.13). For all detected fluorescence values, linear regressions were calculated (Tab. 3.3). Only 23 regressions showed a $R^2 < 0.95$ for about 1000 fluorescence series. This method is applicable even with longer incubation periods.

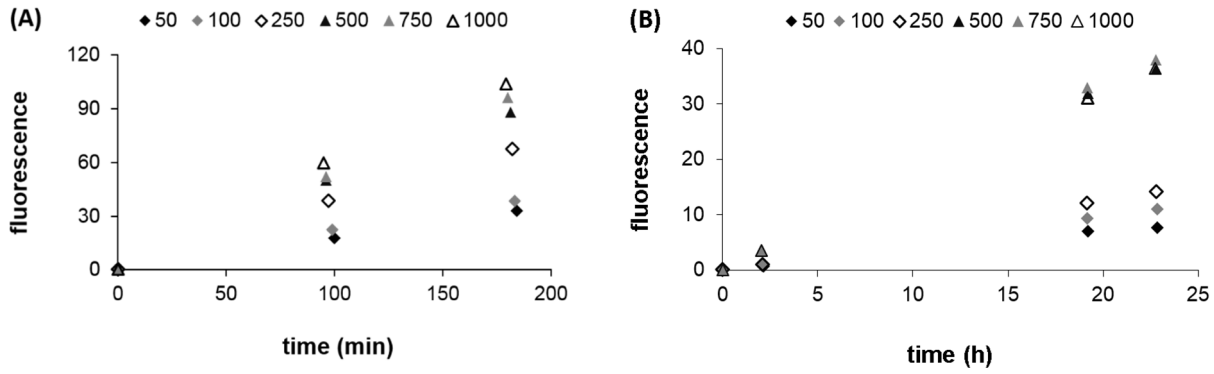


Fig. 3.13: Fluorescence rise of MUF- resp. AMC-fluorophore due to enzymatic cleavage. Fluorescence rise at different MUF-resp. AMC-model substrates (50 – 1000 µmol l^{-1}) for L-Alanine-AMC in October 2009 (A) and MUF-β-D-glucopyranoside in September 2009 (B).

Hydrolytic activities were calculated from the slopes of the fluorescence rise. The standard deviation with 3 – 5 parallels ranged between 0.1 – 65 % (median 15 %) for aminopeptidases and 1 – 74 % (median 13 %) for glycusidases. The hydrolytic activities increased with increasing substrate concentrations. These hydrolytic activities for one enzyme were plotted against the corresponding substrate concentration (Fig. 3.14). At higher substrate concentrations, the hydrolytic activities do not increase longer. Thus, one obtains saturation curves. With these saturation curves Michaelis-Menten kinetic parameters were extrapolated.

Tab.3.3: Linear regression (R^2) for examined enzymes to control linear fluorescence rise of the enzymatically cleaved MUF- resp. AMC- fluorophore. Incubation time, how many times a sample was measured and range of R^2-values with median given in brackets.

Enzyme	Data acquisition	Data points	R^2
α-Glucosidase	5 - 22 h	3 - 4	0.881 – 1 (0.999)
β-Glucosidase	5 - 22 h	3 - 4	0.989 – 1 (0.999)
Cellobiodase	5 - 22 h	3 - 4	0.648 – 1 (0.999)
N-Acetylglucosaminidase	5 - 22 h	3 -4	0.964 – 1 (0.999)
(Leu-) Aminopeptidase	- 4 h	3	0.849 – 1 (0.999)
(Ala-) Aminopeptidase	- 4 h	3	0.904 – 1 (0.999)
(Phe-) Aminopeptidase	- 4 h	3	0.823 – 1 (0.998)
(Arg-) Aminopeptidase	- 4 h	3	0.944 – 1 (0.999)

Theoretical activities for every substrate concentration were calculated after the Michaelis-Menten equation assuming any Km and Vmax parameter. The differences between these "calculated" and "measured" activities were squared (deviation squares). They were also normalised by the corresponding "measured" activity for a better comparability between the kinetics.

The program Solver (Microsoft Excel) finally generated the Michaelis-Menten parameters by adjusting the Vmax- and Km- values that the summed up deviation squares became minimal. These deviation squares show the reliability of the derived Michaelis-Menten paramters. In general, the sums of the normalized deviation squares were lower for the aminopeptidases than the ones for glycosidases (Tab.3.4).

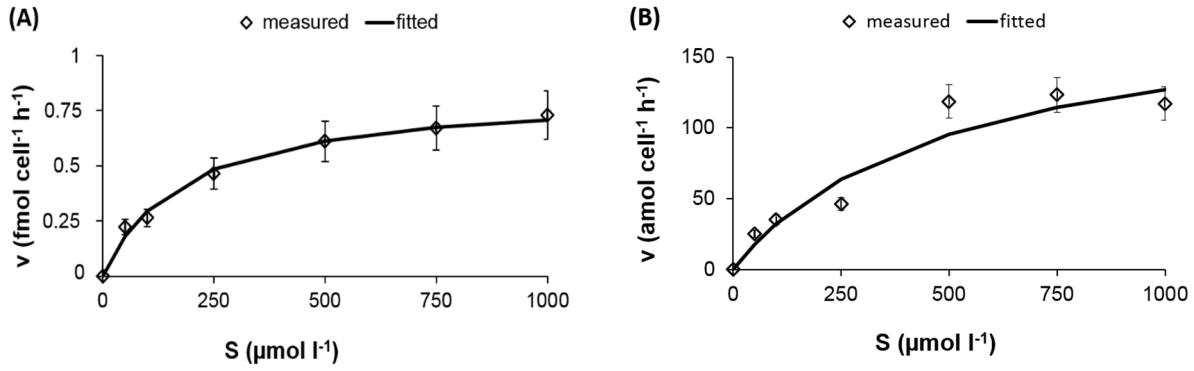


Fig. 3.14: Activity v of Ala-aminopeptidase (A) and β-glucosidase (B) with increasing substrate concentration S. Measured activities (rhombus) are given in mean with standard deviation (n = 3). Measured activities were fitted (black line) with iterative least deviation curve fitting program (Solver in Excel) to obtain Michaelis-Menten parameters Vmax and Km.

Tab. 3.4: Range of normalized deviation square sums for all examined aminopeptiodases (n = 14) and glycosidases (n = 13). Median in brackets.

Enzyme	sum of normalized deviation squares	enzmye	sum of normalized deviation squares
Ala-aminopeptidase	0.01 – 0.03 (0.05)	α-glucosidase	0.4 – 23 (1.8)
Leu-aminopeptidase	0.01 – 0.06 (0.04)	β-glucosidase	0.07 – 23 (8)
Phe-Aminopeptidase	$3.4 \cdot 10^{-5}$ – 0.01 (0.07)	Cellobiodase	0.01 – 11 (0.6)
Arg-Aminopeptidase	$2.4 \cdot 10^{-12}$ – 0.01(0.002)	N-Acetylglucosaminidase	0.01 – 3.5 (0.4)

The sums of the normalized deviation squares showed a mediocre to strong correlation with their corresponding Vmax-values, but just because of some outliers (Fig. 3.15). The correlation with Km-values was weak. No classification for these summed up deviation squares exits. Which values of summed up normalised deviation squares mean that the derived Michaelis-Menten parameters are reliable and which not, is not possible to tell. All in all, higher Vmax- and Km-values did not result in higher sums of normalised deviation squares.

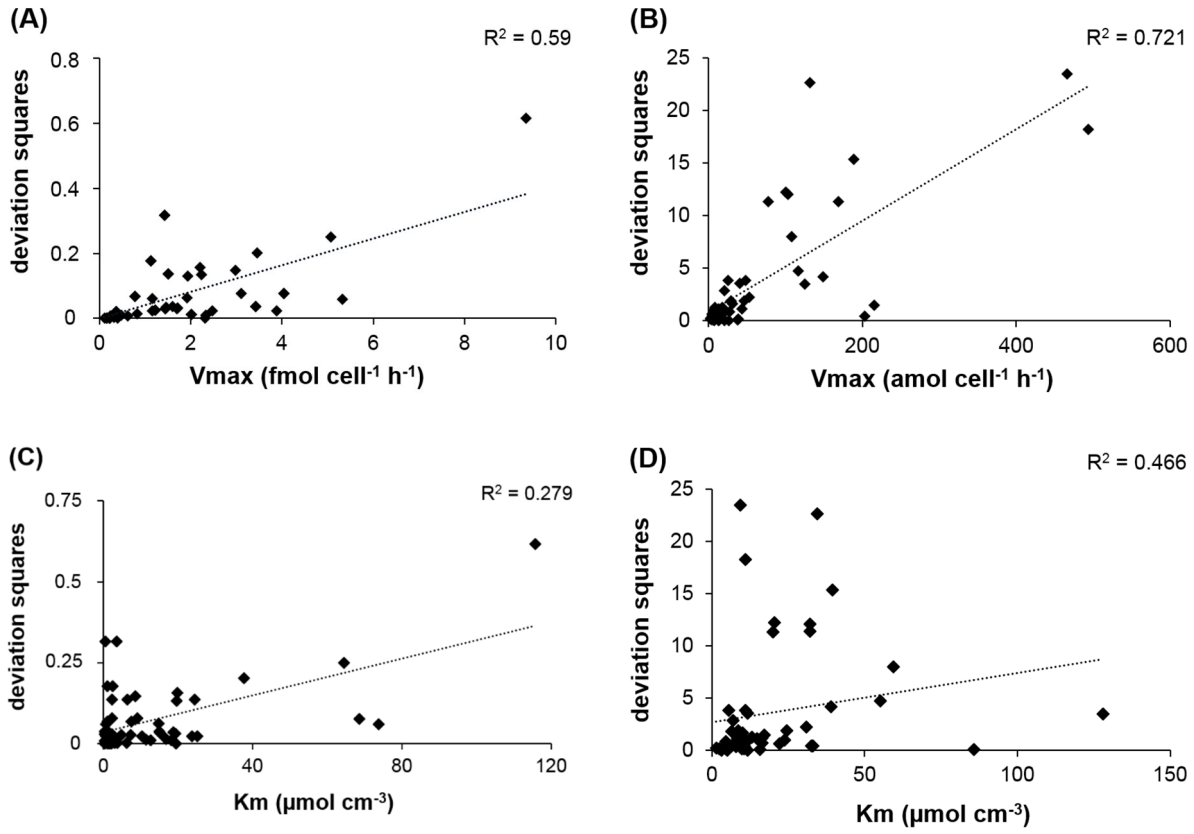


Fig. 3.15: Sum of normalized deviation squares plotted against corresponding Vmax and Km-values of Michaelis-Menten kinetics for aminopeptidases (n = 53) (A & C) and glycosidases (n = 52) (B & D). Spearman-Rho correlation coefficient R^2 in figure.

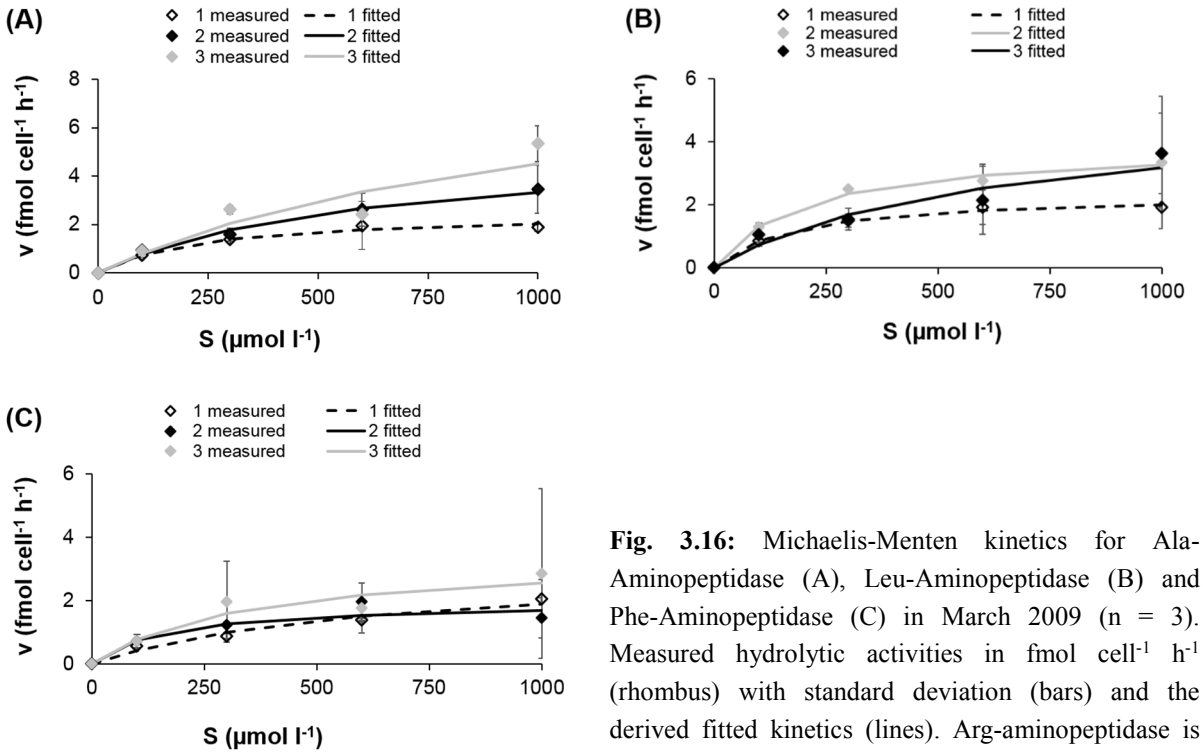


Fig. 3.16: Michaelis-Menten kinetics for Ala-Aminopeptidase (A), Leu-Aminopeptidase (B) and Phe-Aminopeptidase (C) in March 2009 (n = 3). Measured hydrolytic activities in fmol cell^{-1} h^{-1} (rhombus) with standard deviation (bars) and the derived fitted kinetics (lines). Arg-aminopeptidase is lacking because substrate did not suspend.

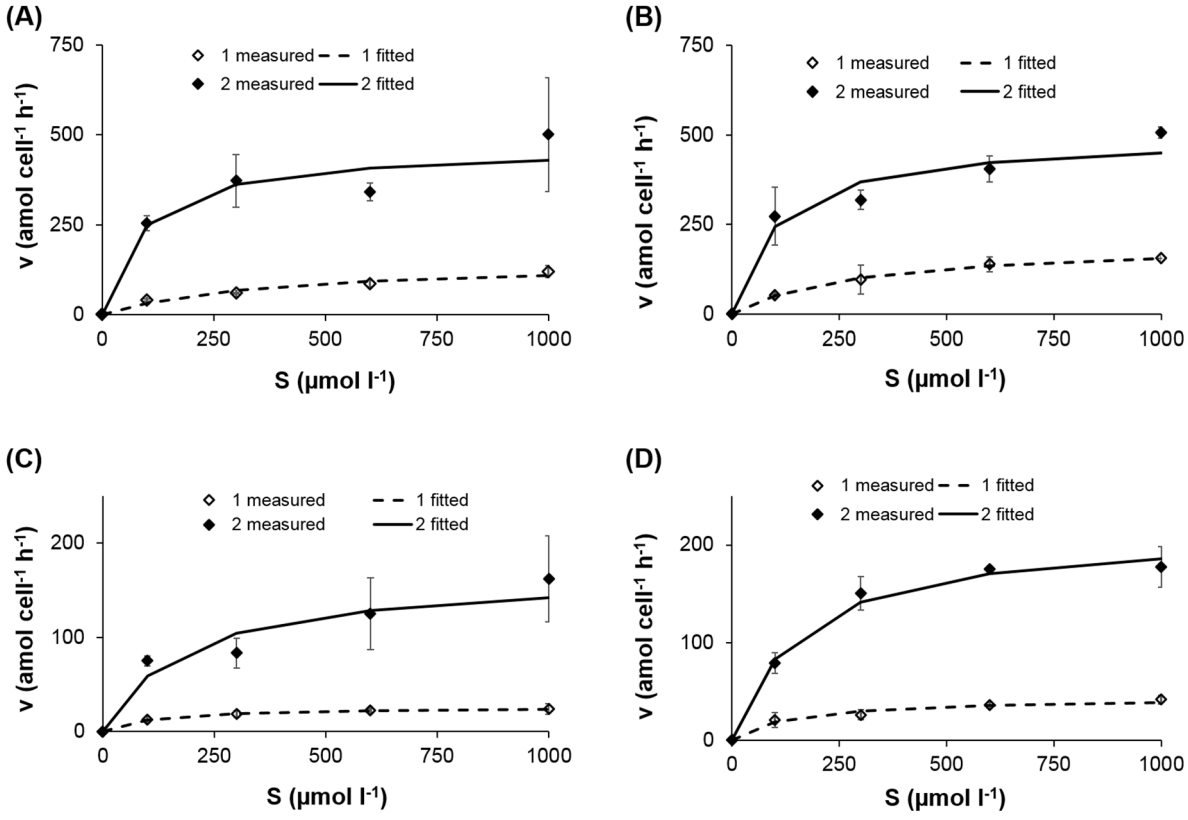


Fig. 3.17: Michaelis-Menten kinetics for α-glucosidase (A), β-glucosidase (B), cellobiodase (C) and N-Acetylglucosaminidase (D) in February 2009 (n = 3). Shown are the measured hydrolytic activities in amol cell^{-1} h^{-1} (rhombus) with standard deviation (bars) and the derived fitted kinetics (lines).

The hydrolytic enzyme activities were measured with a different amount of replicates every month. In general, the aminopeptidases showed a one order of magnitude higher apparent Vmax-value than the glycosidases. The Vmax-values were in the same range between the aminopeptidases and within the corresponding replicates (2 – 9 fmol $cell^{-1}$ h^{-1} in March 2009 (Fig. 3.16).

The glycolytic activities were more heterogenic. The activities ranged between 30 – 500 amol $cell^{-1}$ h^{-1} in February 2009. Vmax- values of α- and β-glycusidase were two to three times higher than of cellobiodase and of N-Acetylglucosaminidase (Fig. 3.17).

The Vmax-values for both aminopeptidases and glycosidases were highest during winter/ early spring. The Vmax-values for Ala-, Leu- and Phe-aminopeptidase were two to three times lower in summer (July 2009) and autumn (October 2009) compared to March 2009 (Fig. 3.18 A). These three aminopeptidases did not differ very much to each other in the ratio of the Vmax-values (1 – 5 amol $cell^{-1}h^{-1}$). Interestingly, the Vmax-value of the Arg-aminopetidase, the amino acid containing proportionally the most nitrogen, was with 0.1 – 0.6 amol $cell^{-1}h^{-1}$ up to ten times lower than the Vmax-values of the other three examined aminopeptidases (Tab 3.5).

As the aminopeptidases were rather stable in their hydrolytic activity, the glycosidases fluctuated much more over the year. The Vmax-values of all four glycosidiases were about 25 - times lower in summer (June 2009) than in winter (February 2009). The Vmax-values increased 1.5 – 8 – times in September 2009. (Fig. 3.18 B). The ratio of the four glycosidases was always the same in February and June 2009. The Vmax-ratio of the four glycosidases was in September 2009 completely different compared to February and June. Vmax-values of the α- and β-glycosidase were on the same level in February and June. Vmax-values of cellobiodase and N-Acetylglucosaminidase were on the same level, but two to three times lower than the ones of α- and β- glycosidase. In September, the Vmax-value of the β-glucosidase was the highest, whereas the Vmax-values of the α-glucosidase and N-Acetylglucosaminidase were on the same level, but four to five times lower than the β-glucosidase. The Vmax of the cellobiodase was the lowest of all four.

The Km-values as indicator for enzymatic substrate affinity were not as different as the Vmax-values between aminopeptidases and glycusidases. Looking at aminopeptidases, the substrate affinity was lowest in March. The substrate affinity increased in July and October (Fig 3.18 C). The Km-values were 4 – 35 times lower than in March. The substrate affinity of the Ala-aminopeptidase was with Km-values of 15 – 80 µmol cm^{-3} the lowest. The substrate affinity of the other aminopeptidases was especially in July and October remarkably higher with Km values between 0.7 – 4 µmol cm^{-3}.

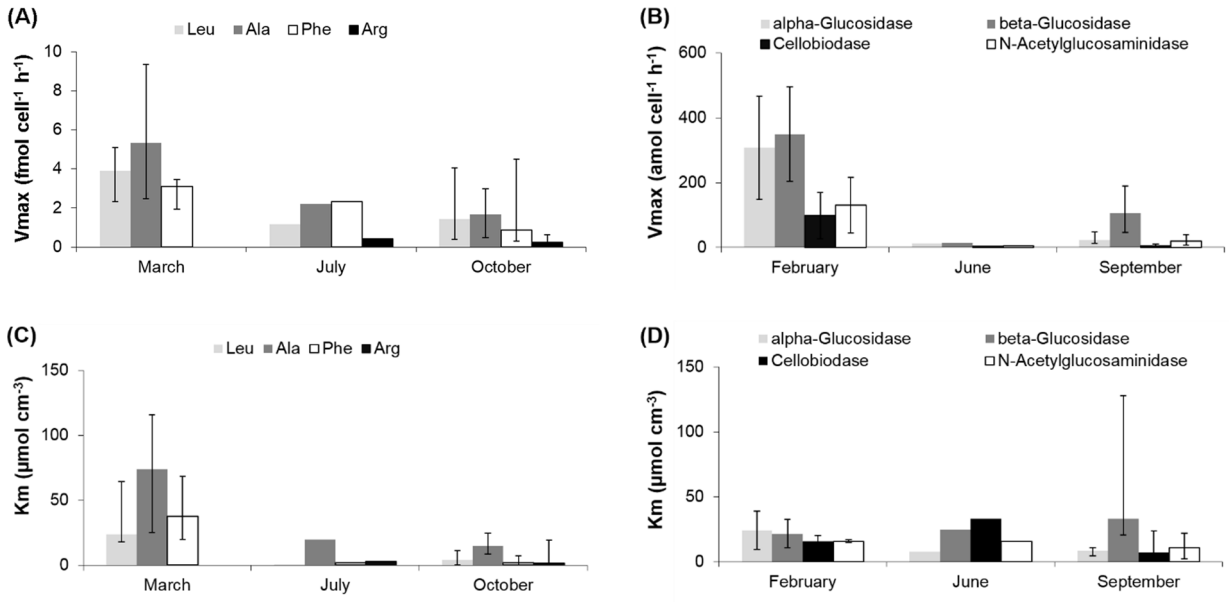


Fig. 3.18: Michaelis-menten parameters for hydrolytic activities in microbial mat during 2009. Vmax in fmol cell^{-1}h^{-1} for aminopeptidases (A), in amol cell^{-1}h^{-1} for glycusidases (B) and Km in µmol cm^{-3} for aminopeptidases (C) and glycosidases (D). Median and minimal and maximal values.

Tab. 3.5: Michaelis – Menten kinetic parameters for Ala-aminopeptidase (Ala), Leu-aminopeptidase (Leu), Phe-aminopeptidase (Phe) and Arg-aminopeptidase (Arg) within the microbial mat in March (n = 3), July (n = 1) and October (n = 10) 2009. Minimal and maximal values for apparent Vmax (fmol cell^{-1} h^{-1}) and apparent Km (µmol cm^{-3}), median in brackets. n.d = no data.

	March		**July**		**October**	
	Vmax	*Km*	*Vmax*	*Km*	*Vmax*	*Km*
Ala	2.5 – 9 (5)	25 – 116 (80)	2	20	0.5 – 3 (2)	8 – 25 (15)
Leu	2 – 5 (4)	18 – 64 (24)	1.2	0.7	0.4 – 4 (1.4)	0.4 – 12 (4)
Phe	2 – 3.5 (3)	20 – 70 (38)	2.4	2	0.3 – 4.5 (0.9)	0.3 – 7 (2)
Arg	n.d.	n.d.	0.4	3	0.1 – 0.6 (0.2)	0.3 – 20 (2)

Tab. 3.6: Michaelis – Menten kinetic parameters for α- and β-glucosidase (α-Gluc./ β-Gluc.), N-Acetylglucosamindase (NAC)and cellobiodase (Cellob.) within the microbial mat in February (n = 2), June (n = 1) and September (n = 10) 2009. Minimal and maximal values for apparent Vmax (amol cell^{-1} h^{-1}) and apparent Km (µmol cm^{-3}), median in brackets

	February		**June**		**September**	
	Vmax	*Km*	*Vmax*	*Km*	*Vmax*	*Km*
α-Gluc.	150 – 470	9 – 40	13	8	10 – 50 (23)	5 – 11 (9)
β-Gluc	200 – 500	11 – 33	14	86	50 – 190 (100)	20 – 60 (33)
NAC	40 – 220	15 – 17	5	16	7 – 40 (20)	2 – 22 (11)
Cellob.	30 – 170	12 – 20	4	33	2 – 11 (6)	1 – 24 (7)

No difference between these three aminopeptidases could be distinguished (Tab. 3.5). The glycosidases did not alternate in their substrate affinity as much as the aminopeptidases over the year 2009 (Fig. 3.11 D). Only the substrate affinity of the β-glucosidase was with 86 µmol cm^{-3} in July remarkably lower than the other three glycosidases. The substrate affinity of the other three glycosidases was with Km-values of 8 – 33 µmol cm^{-3} 2.5 - 10 times higher. The substrate affinity of the four glycosidases was with Km-values between 7 – 40 µmol cm^{-3} in the same range in the other months (Tab. 3.6).

How diverse were these hydrolytic activities distributed within the microbial mat and over the year 2009? The months with 10 replicates represent an area of approximately 1 – 2 m^2 (space) and can be compared with all examined hydrolytic activities over the year (time). The Vmax-value is the parameter for the potential maximal hydrolytic activity of the corresponding enzyme. To compare

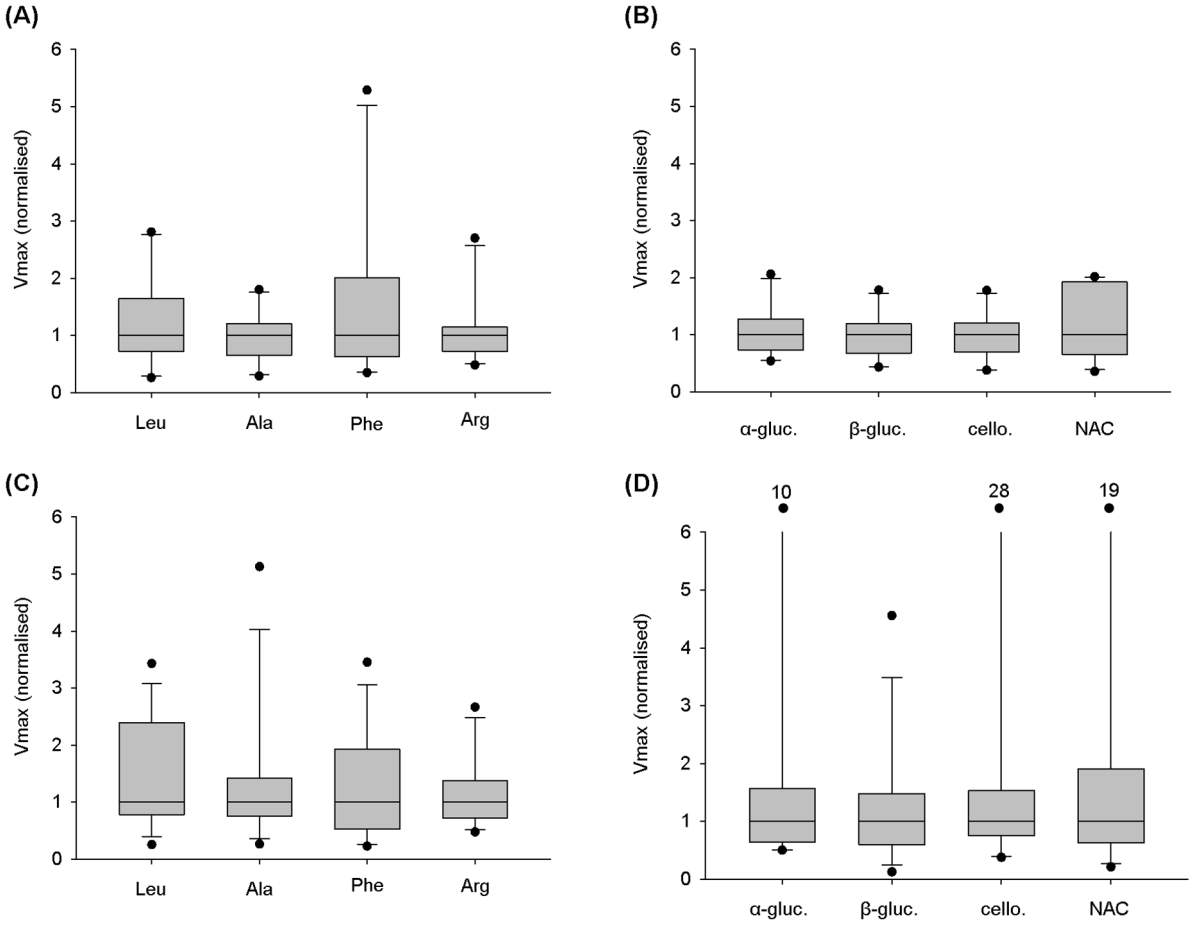


Fig. 3.19: Box-Whisker-plots for apparent Vmax-values of aminopeptidases and glycusidases in microbial mat 2009. Vmax-values are normalized by the corresponding median for better comparability: Vmax of aminopeptidases in October (n = 10) (A), glycusidases in September (n = 10) (B), aminopeptidases (n = 24) (C) and glycusidases (n = 23) (D) over the whole year 2009.

these Vmax-values of the different enzymes, all Vmax-values were normalised by the respective median of the month or year and Box-Whisker-plots were generated. The wider the box, covering 50 % of the values, the more heterogenic the activities were distributed.

Hydrolytic activities of Leu- and Phe-aminopeptidase were more heterogenic than Ala- and Arg-aminopeptidase looking at the spatial distribution (Fig. 3.19 A). α- and β-glycosidase and cellobiodase were all three equally distributed, whereas N-Acetylglucosaminidase was more heterogenic distributed (Fig. 3.19 B).

Aminopeptidases tended to be more heterogenic than glycosidases over the year 2009. However, all glycosidases had very high extreme values (Fig. 3.19 C, D).

3.3 The potential of heterotrophic bacteria to degrade organic material

3.3.1 Phylogeny and selection of isolates

Eight of the nine isolates from the wind flat microbial mat belong to the class γ-Proteobacteria, which are typical and widespread in marine habitats. 3.07 B1 was identified as *Bacillus baekryungensis*. This strain belongs to the Gram$^+$-class Bacilli. Gram$^+$-bacteria are also found in marine habitats, but not very common. Seven of the eight γ-Proteobacteria strain are typical marine geni, *Pseudomonas sp*. (3.07 B2) is ubiquitous.

Tab.2.2: Results of the 16SrDNA sequencing of the isolates; BLAST-response with accession number, sequence length (bp) and identicalness according to data base NCBI.

strain	sequence length (bp)	accession no.	identicalness (%)	BLAST response	Class
9.06 B1	1006	FJ546062.1	99	*Pseudoalteromonas sp.*	γ-Proteobacteria
9.06 B2	1038	AB186498.1	99	*Vibrio sp.*	γ-Proteobacteria
9.06 B3	875	EF685673.1	99	*Marinobacter sp.*	γ-Proteobacteria
9.06 B4	904	AM913917.1	100	*Pseudoalteromonas sp.*	γ-Proteobacteria
9.06 B5	902	EF685673.1	99	*Marinobacter sp.*	γ-Proteobacteria
3.07 B1	929	GQ903402.1	99	*Bacillus baekryungensis*	Bacilli
3.07 B2	1377	AM421975.1	99	*Pseudomonas sp.*	γ-Proteobacteria
3.07 B3	365	CP001252.1	100	*Shewanella baltica*	γ-Proteobacteria
3.07 B4	929	GQ327990.1	99	*Shewanella sp.*	γ-Proteobacteria

Some strains had to be excluded from further experiments due to slow growth and/ or unreliable growth. Strains 9.06 B3, 9.06 B5 grew extremely slow (~ $9 \cdot 10^6$ cells ml^{-1}) after 28 h. 9.06 B2 and 3.07 B1 growth was very unstable. Sometimes they grew, sometimes not. Substrate utilisation was analysed for all strains, except 9.06 B3 and 9.06 B5. The strains of the genus *Pseudoalteromonas* (9.06 B1 and 9.06 B4) and the genus *Shewanella* (3.07 B3 and 3.07 B4) were chosen for enzyme screening. Two strains were chosen for the experiments **A** – **C** based on the screening for constitutive enzymes (s. 2.2.2 and 3.3.3 for further details).

3.3.2 Substrate profiles

4 - 15 of 32 tested compounds in the ECOplates were oxidised by the community (Fig. 3.20; s. appendix for more details).

The community oxidised the most C-sources in May within 48 hours. More carbohydrates than amino acids were oxidised. Polymers glycogen, Tween 40, Tween 80 and α-cyclodextrin were oxidised very fast. The community oxidised 6 additional C-sources within nine days, especially amino acids were oxidised.

Only four C-sources were oxidised in August 2009. No carbohydrates were oxidised, only amino acids and polymers. Eleven additional C-sources were oxidised after 9 days, especially carbohydrates and carboxylic acids, but no additional polymer, like glycogen.

14 C-sources were oxidised by the community within 48 hours in October 2009. 10 C-sources were additionally oxidised within 9 days. As many carbohydrates as amino acids were oxidised within 48 hours. The additional oxidised C-sources after 48 hours were mostly carbohydrates.

Polymers, like Tween 40 and Tween 80 (highmolecular weight polymers) were oxidised very quick by the community in all months.

Seven isolates from the microbial mat were tested for their ability to oxidise 92 different C-sources of the BIOLOG GN2 plate (Fig. 3.21; s. appendix for more details). The isolates 9.06 B2 (*Vibrio sp*.), 3.07 B3 (*Shewanella baltica*) and 3.07 B4 (*Shewanella sp.*) were able to oxidise 40 to 43 compounds and had a rather broad spectrum. The isolates 9.06 B1 (*Pseudoalteromonas sp.*), 9.06 B4 (*Pseudoalteromonas sp.*), 3.07 B1 (*Bacillus baekyrungensis*) and 3.07 B2 (*Pseudomonas sp*.) oxidised only 13 – 23 compounds and, hence, had a rather narrow spectrum.

Three metabolic types could be identified, concerning carbohydrate and amino acid oxidation. All strains oxidised a variety of carbohydrates (6 - 14), but the ability to oxidise amino acids differed among them. Three strains (9.06 B2, 3.07 B3 & 3.07 B4) oxidised many carbohydrates and many amino acids (8 - 10). Two strains (9.06 B1 & 3.07 B1) oxidised many carbohydrates and only a few amino acids (2 - 3). Two strains (9.06 B4 & 3.07 B2) oxidised many carbohydrates and no amino acids at all.

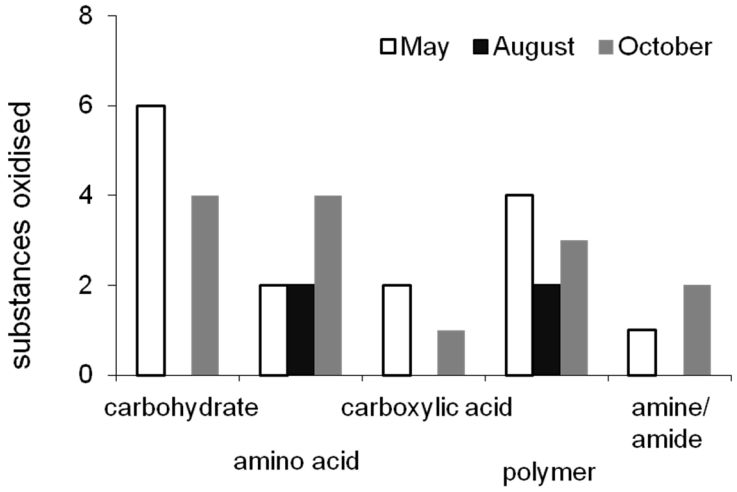


Fig. 3.20: ECOplate profiles for microbial mat community with the number of C-sources oxidised in May, August and October 2009. C-sources are sorted into carbohydrates (10 substrates), amino acids (6), carboxylic acids (9), polymers (4) and amine/ amide (2) according to Weber and Legge (2009). See Appendix for more detailed results. n = 3; incubation time = 48 h, for C-sources respired after 48 h see appendix

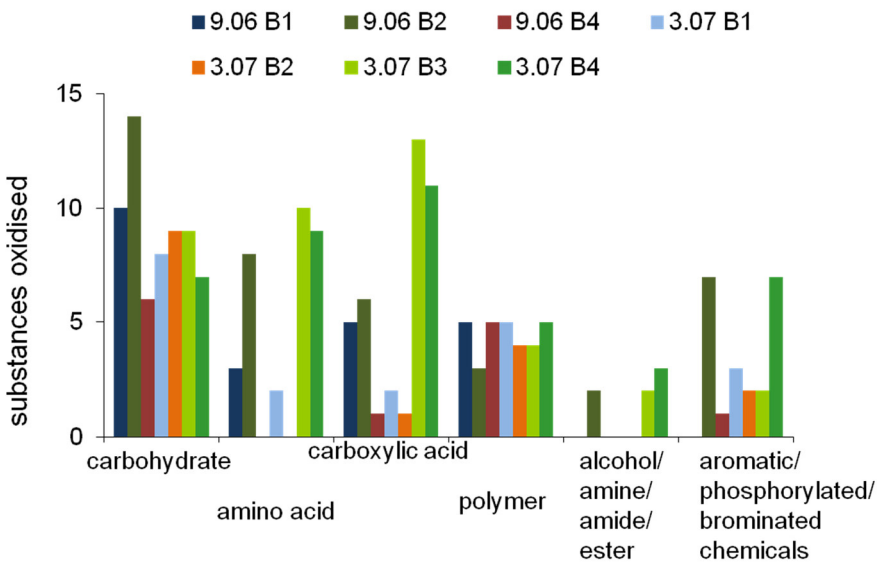


Fig. 3.21: Biolog profiles (GN2) for bacterial strains 9.06 B1, 9.06 B4, 3.07 B3 and 3.07 B4 from wind flat microbial mat (n = 1). Incubation time = 1 h. C-sources are sorted into carbohydrates (28 substrates), amino acids (20), carboxylic acids (24), polymers (5), alcohols/ amine/ amide/ ester (10) and aromatic, phosphorylated and brominated chemicals (8) according to Garland and Mills (1991). See Appendix for more detailed results.

The substrate profiles were slightly different compared with the community substrate profiles as carbohydrates seemed to be oxidised as much as amino acids.
However, N-Acetyl-D-glucosamine was oxidised by six strains, cellobiose by five and the polymer glycogen by six. All strains oxidised very fast the polymers Tween 40 and Tween 80. This corresponded very well with the community respiration patterns.

3.3.3 Hydrolytic activities

The four analysed isolates showed different patterns of apparent constitutive hydrolytic activity (Tab. 3.7). Strain 9.06 B1 (*Pseudoalteromonas* sp) and 3.07 B4 (*Shewanella* sp) expressed all tested hydrolytic activities (α- and β- glucosidase, N-Acetylglucosaminidase and cellobiodase), whereas 9.06 B4 (*Pseudoalteromonas* sp) and 3.07 B3 (*Shewanella baltica*) only showed α-glucosidase and N-Acetylglucosaminidase activity.
Two isolates were chosen for following analyses based on these results: 3.07 B4 showed all tested hydrolytic activities. Furthermore, only 3.07 B4 was able to grow on minimalmedium. Minimalmedium contains only one carbon source. This is important to study the influence of different substrates on bacterial growth, enzyme activity etc.. Thus, 3.07 B4 was chosen and 9.06 B1 which also expressed all tested hydrolytic activities was excluded. 9.06 B4 showed two of four tested hydrolytic activities. 3.07 B3 was excluded because it grew very slowly making the experiments logistically difficult.

Tab. 3.7: Glycolytic activity of four bacterial isolates from wind flat microbial mat after 24 h growth. Glycolytic activity v in fmol $cell^{-1}$ h^{-1}. Substrate concentration was 800 µmol l^{-1}. Mean value and standard deviation (n = 3). n.a. = no activity.

Isolat	**9.06 B1**	**9.06 B4**	**3.07 B3**	**3.07 B4**
Glycolytic activity		**v (fmol $cell^{-1}$ h^{-1})**		
α-glucosidase	1 ± 0.3	1.1 ± 0.4	0.5 ± 0.07	3 ± 0.3
β-glucosidase	1.3 ± 0.3	n.a.	n.a.	8 ± 0.3
cellobiodase	0.3 ± 0.2	n.a	n.a.	0.2 ± 0.003
N-Acetylglucosaminidase	525 ± 14	11 ± 0.3	0.2 ± 0.02	107 ± 1

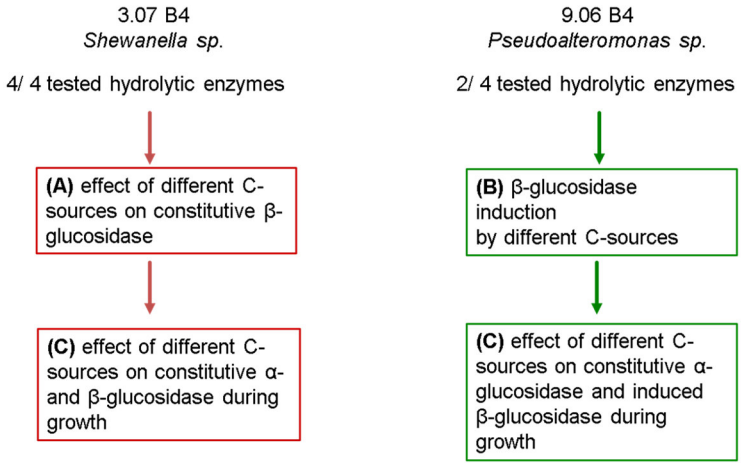


Fig. 3.22: Strategy and tasks of the experiments carried out with isolate 3.07 B4 (constitutive α- and β-glucosidase) and 9.06 B4 (constitutive α-glucosidase).

Three experiments were carried out (Fig. 3.22):

(A) The influence of different C-sources on constitutive β-glucosidase activity (3.07 B4 – *Shewanella* sp).

(B) β-glucosidase induction by different C- sources. (9.06 B4 – *Pseudoalteromonas* sp).

(C) Effect of different C-sources on constitutive hydrolytic enzymes (α- and β-glucosidase) for 3.07 B4 (*Shewanella* sp) and on constitutive (α-glucosidase) and inducible (β-glucosidase) hydrolytic enzymes for 9.06 B4 (*Pseudoalteromonas* sp) during 10 days growth.

A. 3.07 B4 (*Shewanella* sp) was grown on seawater yeast peptone (SYP)-medium and minimalmedium containing cellobiose, starch or glucose in 0.5 % (mass percentage) (Fig. 3.23). SYP-medium and minimalmedium with glucose contain compounds easily utilizable by the bacteria. Hence, the bacteria on these media grew faster than on the other two media (Tab. 3.8). Bacteria on SYP-medium had with 19 ng BSA-equivalents $cell^{-1}$ a 7- to 60-times higher protein content due to the protein and peptides in the medium. Bacteria grew slower on minimalmedium with cellobiose as it is harder to access by the bacteria than peptone and glucose. Bacteria did not grow on the minimalmedium with starch in this approach as starch is a polymer not easily degradable.

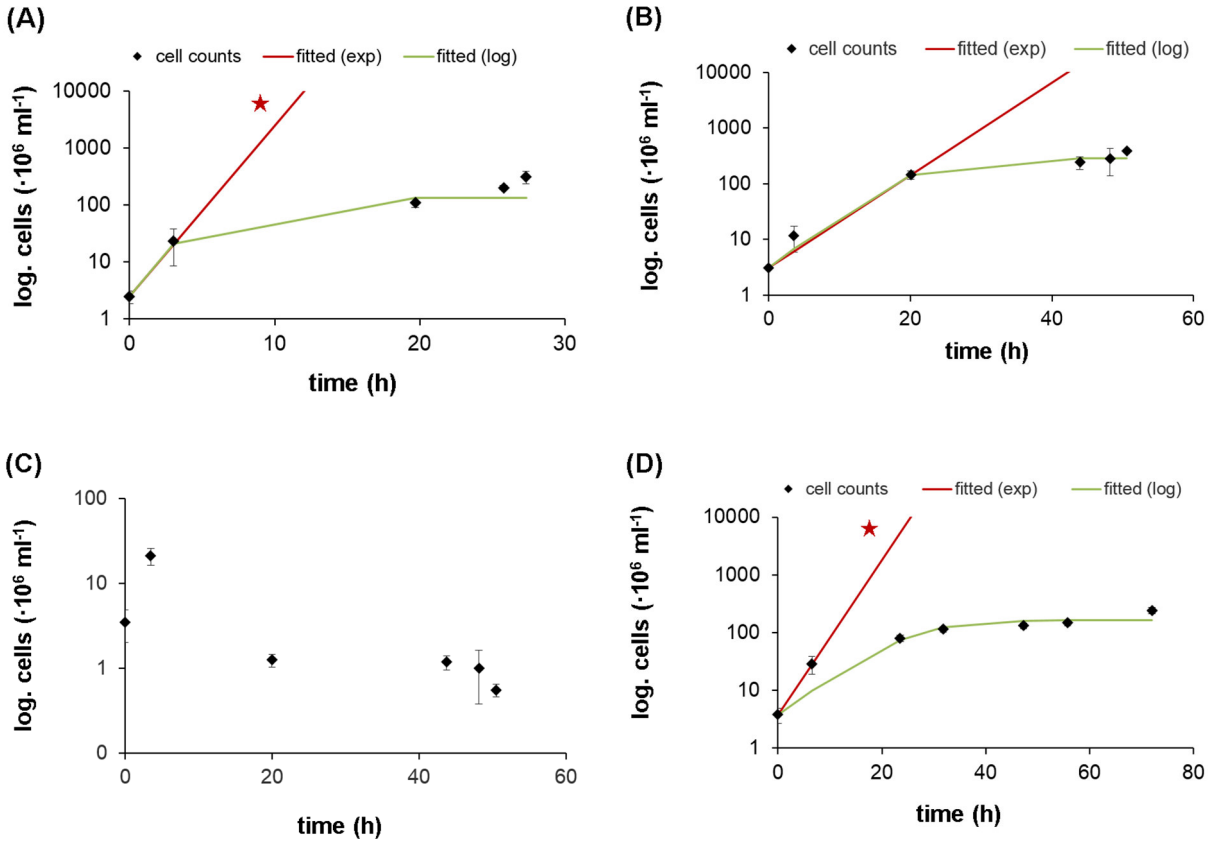


Fig. 3.23: Growth of isolate 3.07 B4 on SYP-medium (A) and minimalmedium with additional carbon sources of 0.5 % (mass percentage rate) cellobiose (B), starch (C) and glucose (D) for 30 – 72 h. Growth in cell counts in cells · 10^6 ml^{-1} (black rhombus) and growth rates fitted with model of exponential growth (fitted (exp) – red line) and model of logistical growth (fitted (log) – green line). ★Only cell numbers of t(0) and t(1) were used for exponential fit n = 3, mean and standard deviation.

The β-glucosidase activity was lowest (~ 1 amol $cell^{-1}$ h^{-1}) on SYP-medium and minimalmedium with glucose. The β-glucosidase activity was 13-times higher on minimalmedium with the disaccharide cellobiose and 65-times higher on the minimalmedium with starch. This is interesting as bacteria did not grow on the starch-containing medium. Furthermore, starch consists of α-1,4- and α-1,6-glycosidic bonds (Fig. 3.24) only degradable by α-gluosidase.

Tab. 3.8: Growth parameters of isolate 3.07B4 on different media with additional carbon souces of 0.5 % (mass percentage rate) calculated with model of exponential growth and logistical growth. Growth rate μ in h^{-1} doubling time dt in h and capacity K in cells · 10^6 ml^{-1}, mean value. M = minimal medium, n.g. = no growth (n = 3)

	exponential growth		logistical growth		
medium	**μ (h^{-1})**	**dt (h)**	**μ (h^{-1})**	**K (cells · 10^6 ml^{-1})**	**dt (h)**
SYP	0.69	1	0.75	130	0.9
M + cellobiose	0.19	3.6	0.23	290	3.1
M + starch	n.g.		n.g		
M + glucose	0.31	2.3	0.15	160	4.6

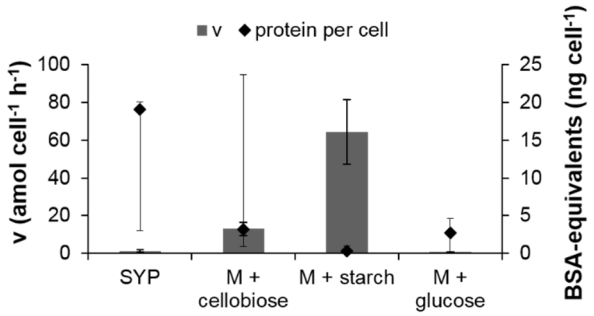


Fig. 3.24: Constitutive β-gylcolytic activity and protein content for isolate 3.07 B4. on peptone-containing medium and minimalmedium (MiMA) with 0.5 % (mass percentage rate) cellobiose, starch and glucose as only C-source for 24 – 72 h. β-glcolytic activity v in amol cell^{-1} h^{-1} measured after 20 h growth (peptone-medium) and 44 – 48 h (minimalmedia). Substrate concentration was 750 µmol l^{-1}; protein content in BSA-equivalents (ng cell^{-1}). Median and minima and maxima values (n = 3).

B. 9.06 B4 (*Pseudoalteromonas* sp) was grown on SYP-medium with cellobiose and starch given extra in 0.5 % (mass percentage rate), as the isolate did not grow on minimalmedium.

Bacteria grew ~ 2 times faster on the SYP-medium with cellobiose (Tab. 3.9). Interestingly, lag-phase lasted 24 hours on this medium. It only lasted 6 hours on when bacteria were grown on SYP-medium with starch (Fig. 3.25).

Cellular protein content and β-glucosidase activity rose steadily when bacteria were grown on SYP-medium with cellobiose. No β-glucosidase activity was measurable after 24 h due to less than 1 · 10^6 cells ml^{-1} (Fig. 3.26 A).

Cellular protein content on the SYP medium with starch was highest after 48 h and dropped down after 72 h. In general, cellular protein content of 9.06 B4 (*Pseudoalteromonas* sp) was one order of magnitude lower than the one of 3.07 B4.

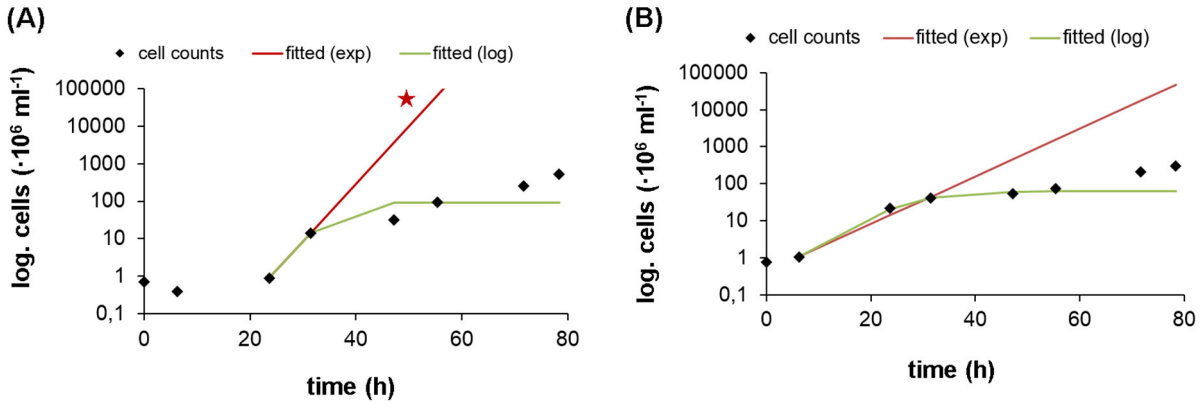


Fig. 3.25: Growth of isolate 9.06 B4 on SYP medium with 0.5 % (mass percentage rate) cellobiose (A) and starch (B) for 80 h. Growth in cell counts in cells · 10^6 ml^{-1} (black rhombus) and growth rates fitted with model of exponential growth (fitted (exp) – red line) and model of logistical growth (fitted (log) – green line). ★Only cell numbers of t(0) and t(1) were used for exponential fit. n =1

Tab. 3.9: Growth parameters of isolate 9.06 B4 on SYP medium with additional carbon souces of 0.5 % (mass percentage rate) calculated with model of exponential growth and logistical growth. Growth rate μ in h^{-1} doubling time dt in h and capacity K in cells · 10^6 ml^{-1}. n = 1.

	exponential growth		logistical growth		
medium	**μ (h^{-1})**	**dt (h)**	**μ (h^{-1})**	**K (cells · 10^6 ml^{-1})**	**dt (h)**
SYP + cellobiose	0.35	2	0.37	92	1.9
SYP + starch	0.15	4.7	0.19	63	3.6

β-glucosidase activity was not as high as the β-glucosidase activity found on SYP-medium with cellobiose. It was three times lower after 48 hours and even thirteen times lower after 72 hours. However, β-glucosidase activity was induced by starch in the medium although it is not the original substrate for the β-glucosidase (Fig. 3.26 B).

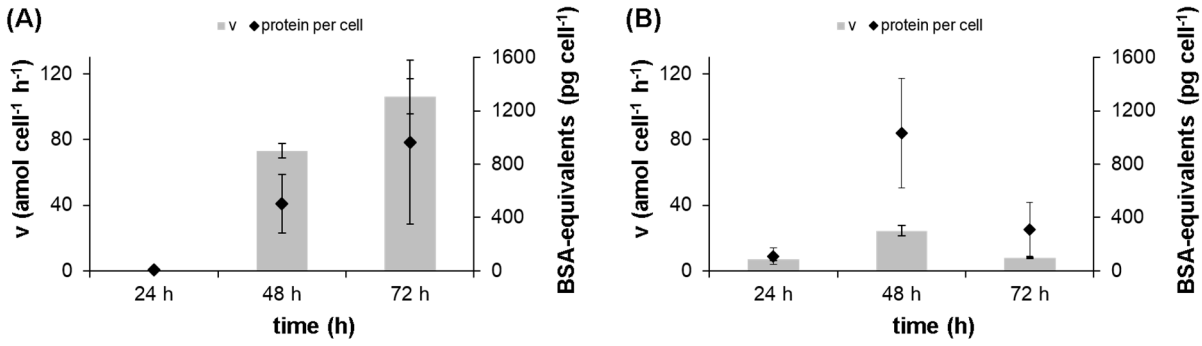


Fig 3.26: Induced β-glycolytic activity and protein content for 9.06 B4 on SYP + cellobiose (A) and SYP + starch (B) containing medium for 80 h incubation. β-glycolytic activity v in amol $cell^{-1}h^{-1}$ and protein content in BSA-equivalents (pg $cell^{-1}$) after 24, 48 and 72 h growth. Substrate concentration was 800 μmol l^{-1}. Mean value and standard deviation (n = 3).

C. Both isolates grew differently and reached the different growth phases at different times. Bacteria in nature are most of the time in stationary growth phase when adopted to the system. A change in amount and kind of substrates available can lead to enhanced growth Thus, it was important to cover exponential as well as stationary growth phase. A "long-time" experiment was carried out for 10 days. Both isolates were cultivated on several media with different substrates added. α-glucosidase activity was measured almost every day. The same was carried out for β-glucosidase activity which was constitutively for 3.07 B4 (*Shewanella* sp) and inducible for 9.06 B4 (*Pseudoalteromonas* sp).

3.07 B4 (*Shewanella* sp) grew very fast on all media and reached stationary growth phase after day 1 (Fig. 3.27). Cell densities were higher on SYP-medium than on minimalmedium with just one carbon source. They ranged from 0.9 – 2.5 cells · 10^9 ml^{-1} on SYP-medium and from 0.1 – 0.2 cells · 10^9 ml^{-1} on minimalmedium (Tab.3.10).

Tab. 3.10: Growth parameters of isolate 3.07 B4 on different media with additional carbon souces of 0.5 % (mass percentage rate) calculated with model of exponential growth and logistical growth. Growth rate μ in h^{-1} doubling time td in h and capacity K in cells · 10^9 ml^{-1}. n = 2, median. M = minimal medium.

	exponential growth		logistical growth		
medium	**μ (h^{-1})**	**td (h)**	**μ (h^{-1})**	**K (cells ·10^9 ml^{-1})**	**dt (h)**
SYP	0.15	4.5	0.12	0.9	5.6
SYP + cellobiose	0.17	4	0.13	1.7	5.2
SYP + starch	0.18	3.9	0.17	1.9	4
SYP + glucose	0.17	4.1	0.12	2.5	5.8
M + cellobiose	0.13	5.4	0.67	0.15	1
M + starch	0.11	6.3	0.71	0.1	1
M + glucose	0.12	5.7	0.23	0.2	3.1

α- and β-glucosidase activites developed different on all media, although all activities were measured during stationary growth phase. Both enzyme activities rose until day 5 and dropped down in the late exponential phase on SYP-medium (Fig. 3.28 A). Both enzyme activities were higher on SYP-medium with cellobiose than on SYP-medium, but only between day 2 – 4 (3 – 10-fold) (Fig. 3.28 B). On SYP-medium with starch, α- and β-glucosdiase activities developed like on normal SYP-medium until day 6. α-glucosidase activity was up to 400 times higher on day 9 and 10 compared with α-glucosidase activity on SYP medium at these days (Fig. 3.28 C). Both enzyme activities were between two and four times lower on SYP-medium with glucose than on SYP medium (Fig. 3.28 D).

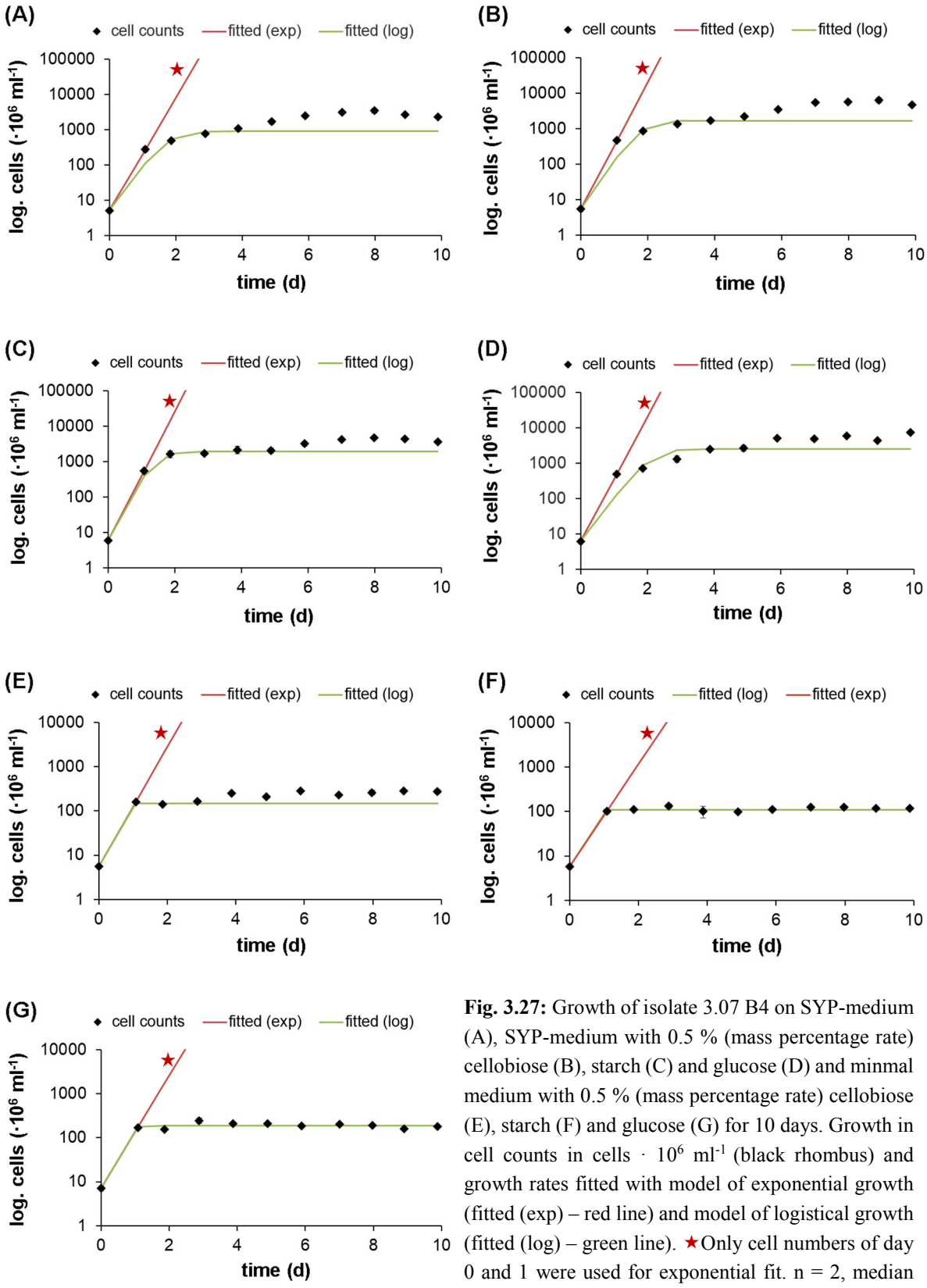


Fig. 3.27: Growth of isolate 3.07 B4 on SYP-medium (A), SYP-medium with 0.5 % (mass percentage rate) cellobiose (B), starch (C) and glucose (D) and minmal medium with 0.5 % (mass percentage rate) cellobiose (E), starch (F) and glucose (G) for 10 days. Growth in cell counts in cells · 10^6 ml^{-1} (black rhombus) and growth rates fitted with model of exponential growth (fitted (exp) – red line) and model of logistical growth (fitted (log) – green line). ★Only cell numbers of day 0 and 1 were used for exponential fit. n = 2, median and minima and maxima.

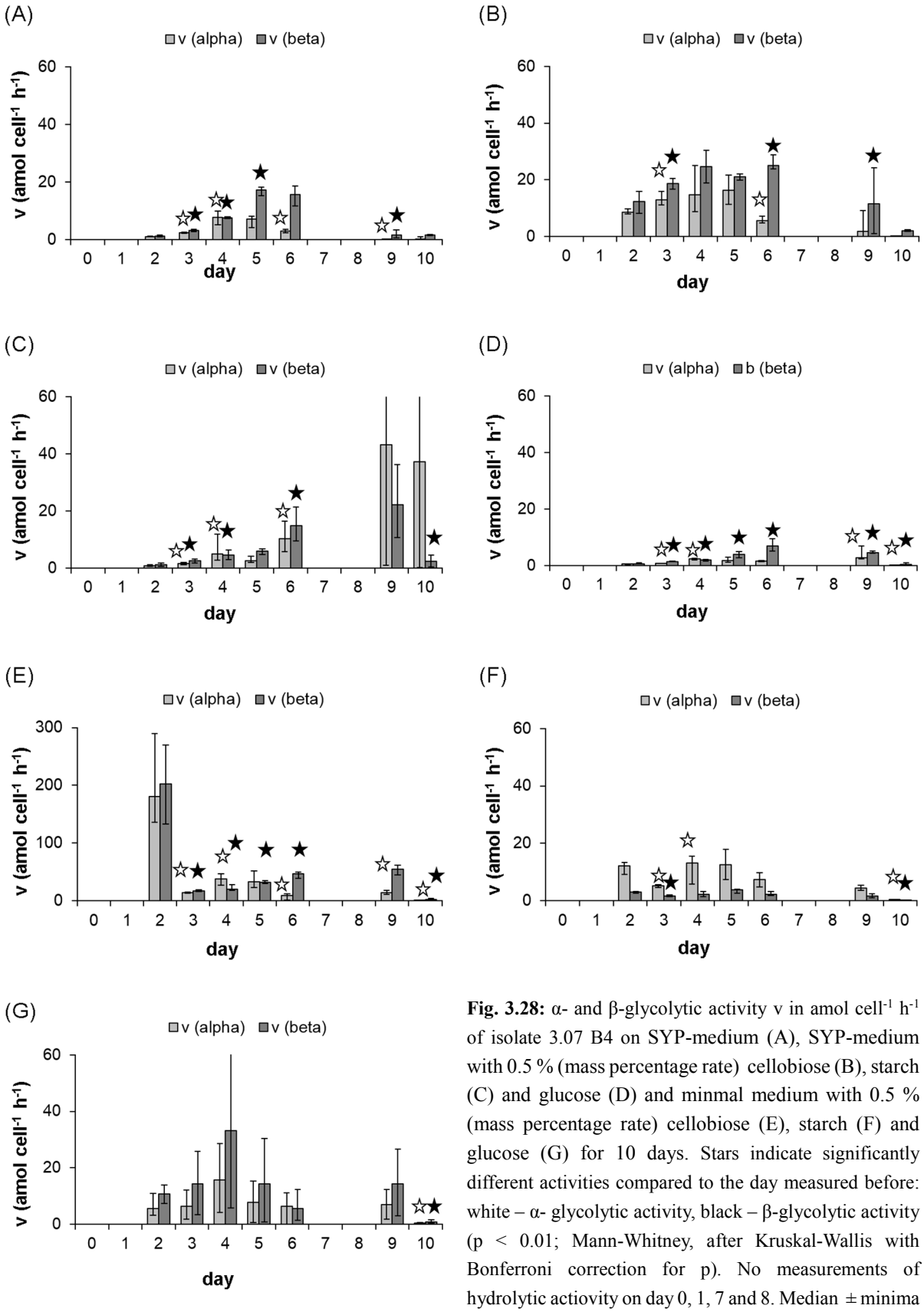


Fig. 3.28: α- and β-glycolytic activity v in amol cell^{-1} h^{-1} of isolate 3.07 B4 on SYP-medium (A), SYP-medium with 0.5 % (mass percentage rate) cellobiose (B), starch (C) and glucose (D) and minmal medium with 0.5 % (mass percentage rate) cellobiose (E), starch (F) and glucose (G) for 10 days. Stars indicate significantly different activities compared to the day measured before: white – α- glycolytic activity, black – β-glycolytic activity ($p < 0.01$; Mann-Whitney, after Kruskal-Wallis with Bonferroni correction for p). No measurements of hydrolytic actiovity on day 0, 1, 7 and 8. Median ± minima and maxima (n = 2).

α- and β-glucosidase activity were in the same range on minimalmedium with just one carbon source as on the peptone containing SYP-medium (0.1 – 40 amol cell^{-1} h^{-1}) . Only on minimalmedium with cellobiose, both enzyme activities were on day 2 (early stationary phase) with 180 – 200 amol cell^{-1} h^{-1} by far the highest (Fig. 3.28 E). The activities dropped down significantly on day 3 and stayed on that level for the rest of the days. However, the activities were still a little bit higher compared with the other media. α-glucosidase activity was three to six times higher than β-glucosidase activity on minimalmedium with starch (Fig. 3.28 F). Both activities were in the same range on SYP-medium with starch.

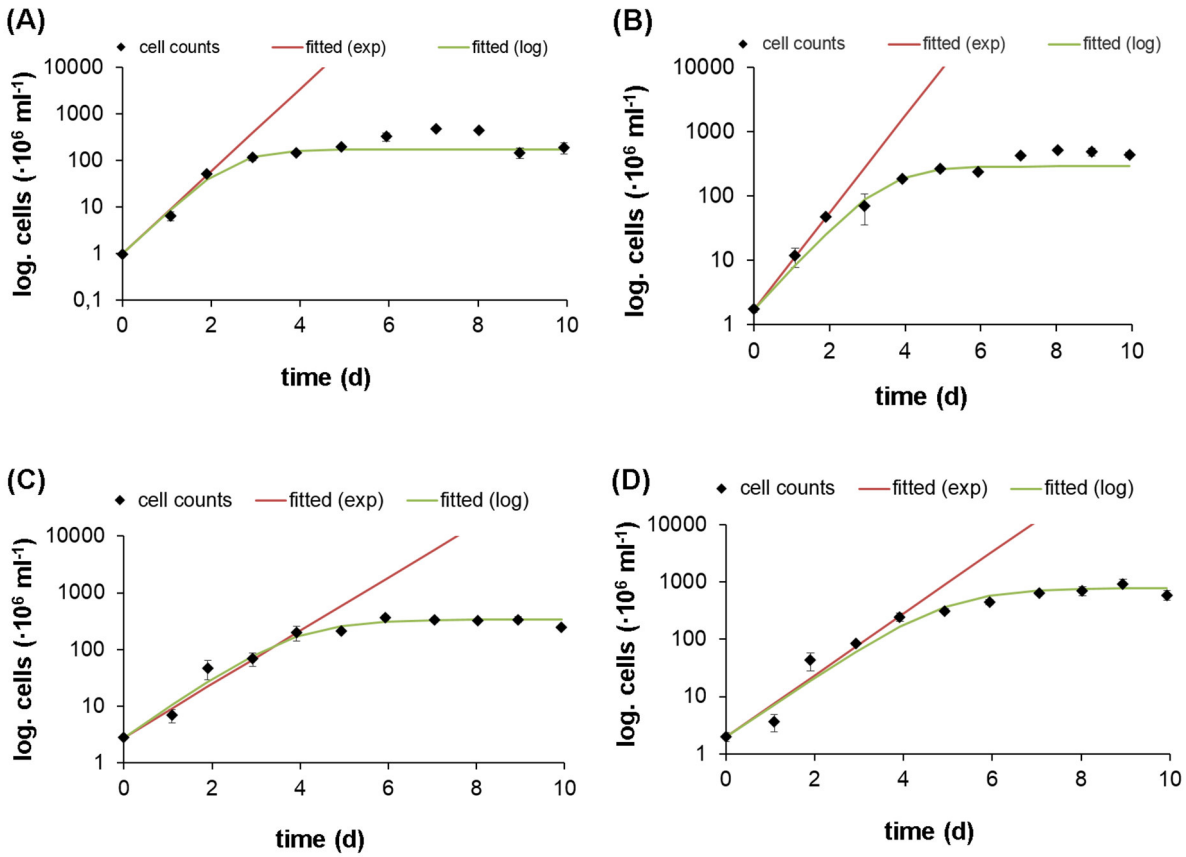


Fig. 3.29: Growth of isolate 9.06 B4 on SYP-medium (A) and SYP-medium with 0.5 % (mass percentage rate) cellobiose (B), starch (C) and glucose(D) for 10 days. Growth in cell counts in cells · 10^6 ml^{-1} (black rhombus) and growth rates fitted with model of exponential growth (fitted (exp) – red line) and model of logistical growth (fitted (log) – green line). n = 2, median and minima and maxima.

9.06 B4 (*Pseudoalteromonas* sp) grew slower than 3.07 B4 (*Shewanella* sp) on the SYP-media (Tab. 3.11). Growth was fastest on SYP-medium. The exponential phase duration was different on the respective media: it lasted until day 2 on SYP- and SYP-medium with cellobiose and until day 4 on SYP-medium with starch and glucose (Fig. 3.29). Thus, hydrolytic activities were measured in exponential and stationary growth phase.

Tab. 3.11: Growth parameters of isolate 9.06 B4 on different media with additional carbon souces of 0.5 % (mass percentage) calculated with model of exponential growth and logistical growth. Growth rate μ in h^{-1}, doubling time dt in h and capacity K in cells · 10^6 ml^{-1}. n = 2, median.

	exponential growth		logistical growth		
medium	**μ (h^{-1})**	**dt (h)**	**μ (h^{-1})**	**K (cells · 10^6 ml^{-1})**	**dt (h)**
SYP	0.085	8.1	0.086	170	8.1
SYP + cellobiose	0.072	9.6	0.061	290	11.4
SYP+ starch	0.045	15.4	0.051	340	13.6
SYP+ glucose	0.051	13.4	0.050	770	14

α- and β-glycolytic activities developed more differently on the different media in contrast to 3.07 B4. α- and β-glucosidase activities were more different from each other on the respective media. α-glycolytic activity was very low on SYP-medium. No activity was measured on day 3 and 5. α-glycolytic activity rose significantly on day 10 (Fig. 3.30 A). α-glycolytic activity was also very low on SYP with cellobiose medium. β-glucosidase activtiy was 100 – 260 times higher than α-glucosidase activity during exponential growth phase on this medium. This ratio became smaller every day as the β-glucosidase activity dropped down during stationary growth phase (Fig. 3.30 B). α-glucosidase was highest in SYP-medium with starch. It rose steadily during exponential growth phase until day 5 and declined during stationary growth phase. β-glucosidase activity again was much higher than α-glucosidase activity, but only up to 15 times higher (Fig. 3.30 C). α-glucosidase activity was not inhibited on SYP with glucose medium. It rose until day 6 (early stationary phase). Also it was up to 170 times higher than on SYP medium and on the same level as on SYP with starch medium (Fig. 3.30 D).

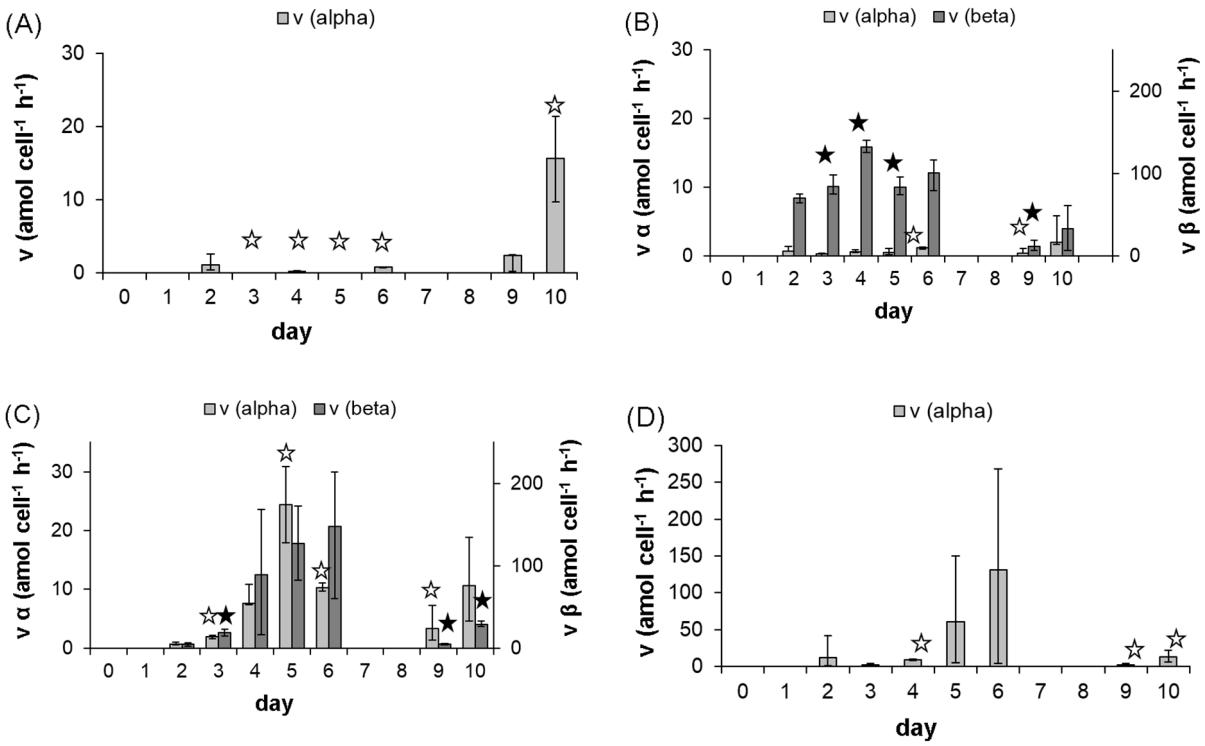


Fig. 3.30: α- and β-glycolytic activity v in amol cell^{-1} h^{-1} of isolate 9.06 B4 on SYP-medium (A) and SYP-medium with 0.5 % (mass percentage rate) cellobiose (B), starch (C) and glucose(D) for 10 days. Stars indicate significantly different activities compared to the day measured before: white – α- glycolytic activity, black – β-glycolytic activity ($p < 0.01$; Mann-Whitney, after Kruskal-Wallis with Bonferroni correction for p). No measurements of hydrolytic actiovity on day 0, 1, 7 and 8. Shown are median ± minima and maxima (n = 2).

4. Discussion

4.0 Methodological considerations

4.0.1 Michaelis – Menten kinetics of apparent hydrolytic activity

Organic material degradation and transformation are microbial key processes in aquatic ecosystems (Wobus et al., 2003). Hydrolytic enzymes are widely used to assess the efficiency and ability of microbial communities to degrade organic matter. However, the interpretation, reliability and usefullness of apparent hydrolytic activities are intensively debated. There are following difficulties:

First of all, it is necessary to think about the localisation of the enzyme. Hydrolytic enzyme activities are generally regarded as extracellular activities. However, most of the released hydrolytic enzymes stay cell-bound (e.g. Schumann et al., 2003). Nearly 70 % of the Leu-aminopeptidase was located in the periplasma within a marine Gram$^-$ bacterium (Martinez and Azam, 1993). More than 98 % of α- and β- Glucosidase and Leucine-Aminopeptidase were cell bound in isolates of γ-Proteobacteria and Cytophaga-Flexibacter-Bacteroides from bioreactors (Konopka and Zakharova, 2002). The substrates are hydrolysed relatively close to the cell to avoid diffusional losses (Martinez and Azam, 1993).

Secondly, one should carefully regard the information gained by hydrolytic activities. Hydrolytic activities are used in aquatic microbiology to gain several insights into the microbial communities' ability to degrade organic matter:

- How efficient is the community to degrade organic matter?
- How is the organic material pool composed as indicated by hydrolytic activities?

Most important, MUF – and AMC-substrates compete with the natural substrates for the active sides of the enzymes. Hence, working with MUF - and AMC-substrates above the saturation limit is essential. Hoppe (1983) and Hoppe et al. (1988) were among the first ones who used MUF- and AMC- substrates in aquatic field studies. They made Michaelis-Menten kinetics with substrate concentrations of 0.5 – 40 µmol l^{-1} and 0.1 – 250 µmol l^{-1}. The saturation level was always reached in these experiments. These settings became general standards in aquatic field studies. Since then, most researches use substrate concentrations of 100 – 250 µmol l^{-1} when working with MUF- and AMC-substrates, assuming that they measure enzyme activities above the substrate saturation level. This may be true for most aquatic ecosystems, like the Darß-Zingst Bodden Chain where Km-values of 150 µmol l^{-1} for β-glucosidase, 200 µmol l^{-1} for Acetate-esterase and 60 µmol l^{-1} for Leu-aminopeptidase were found. The Km-values were higher within the wind flat microbial mat. Glycusidase Km-values up to 400 µmol l^{-1} and aminopeptidase Km-values up to 800 µmol l^{-1} were found for bacterial abundances between 0.06 – 2 · 10^9 cells l^{-1}. 75 % of the glycusidase Km-values

were below 230 µmol l^{-1} and 75 % of the aminopeptidases below 160 µmol l^{-1}. Unfortunately, Michaelis-Menten kinetics in field studies are rare since the pioneer work of Hoppe in the 1980s. Many measurements with one substrate concentration are applied without any knowledge about the natural substrate concentration. A lot of hydrolytic enzyme activities with one substrate concentration were measured in the southern Baltic Sea in the 1980 – 1990s (by Meyer-Reil, Köster etc.). These analyses were used to compare the eutrophication status at different stations.

These single-substrate methods reflect a potential hydrolysis rate, not the absolute amount of enzymes. With any given hydrolytic activity, it is not possible to distinguish between a high catalytic efficiency and a high amount of enzymes (Arnosti et al., 1998). Michaelis-Menten kinetics allow a better comparability of different hydrolytic activities, as the derived kinetic parameters are much more profound than single-substrate measurements.

4.0.2 An approach to use Michaelis - Menten kinetics to balance organic material turnover

To assess how efficient hydrolytic enzymes turn over organic material within the wind flat microbial was one aim of this study. Turnover times for organic material is analysed by measuring uptake and hydrolysis of model-substrates, like Leucine, at the same time in other studies (e.g. Hoppe et al., 1988). Just the Michaelis-Menten parameters were used to gain turnover times (dividing Km by Vmax) in another approach (e.g. Unanue et al., 1999). Both approaches do not take into account the actual organic material amount and composition in the field. Therefore, it is crucial to know the actual organic material amount and its composition. Only microbial mat organic carbon, amino acids and hence proteins were analysed in this study. Literature data and findings for other organisms and ecosystems are needed to calculate the organic material turnover, as the data set of the organic material composition in the wind flat microbial mat is incomplete.

It was difficult to find literature with suiting data to calculate. Thus, studies with a broad acceptance (C-content of bacteria and primary producers (Simon and Azam, 1989)) and studies spatial close to the wind flat (protein and carbohydrate content of POC (Schumann et al., 2001)) were used. The primary producer protein content could only be assessed with data of an organism which is not at all typical for microbial mats because no better data are available right now. Thus, the gained turnover times are a first estimation, although with some insecurities.

The organic material turnover by hydrolytic enzymes can be calculated in two ways: the organic content can be taken as the base for the organic material amount (Fig. 4.1 A) (**A**). The amount of protein/ peptides and carbohydrates based on organic carbon in the microbial mat can be defined with the help of literature data. This way is a more general estimation.

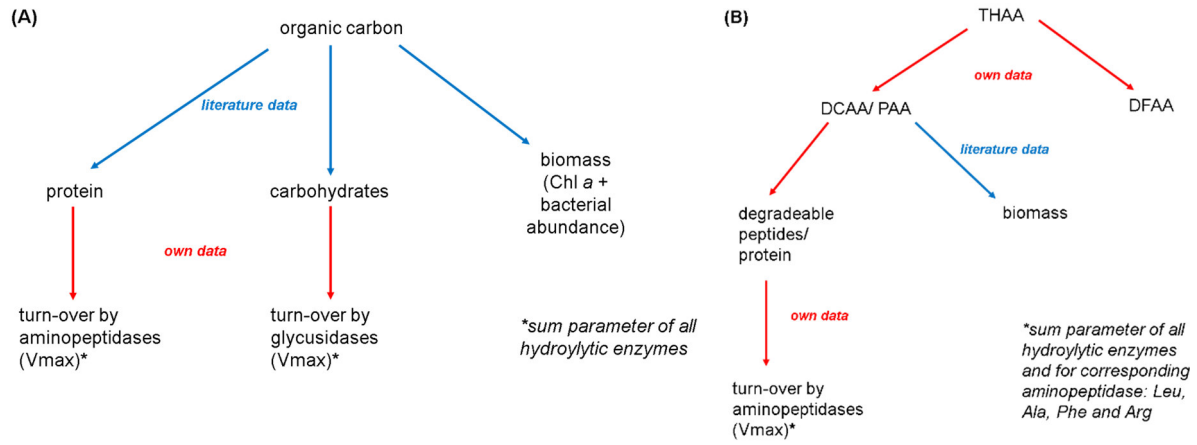


Fig. 4.1: Overview about the two ways in which turnover of peptides and carbohydrates in wind flat microbial mat were calculated. One is based on the organic carbon content in the mat (A), the other on the amino acids analyses in the microbial mat (B). Literature data (blue arrows) were used together with own data for the microbial mat (red arrows) for calculations. THAA = total hydrolysable amino acids, PAA = particulate amino acids, DCAA = dissolved combined amino acids, DFAA = dissolved free amino acids.

The turnover of amino acids based on amino acid analyses within the microbial mat is calculated in a more detailed way for the corresponding amino acid pool and the aminopeptidase degrading it (Fig 4.1 B) (**B**). (a detailed described way of all these calculations can be found in the appendix).

A. The microbial mat organic content of 0.6 - 5 % dry mass is equivalent to 0.5 – 15 mmol org. C cm^{-3}. The organic carbon bound in biomass (primary producers and bacteria) accounts for 0.3 – 0.6 mmol org. C cm^{-3}. This leaves 0.3 – 4 mmol org. C cm^{-3} which are potentially free and degradeable by hydrolytic enzymes (Tab. 4.1). The organic carbon bound in biomass can be negotiated from turnover calculation assuming that proteins and carbohydrates in biomasses are storage- and/ or structural cell components and do not have to be broken down. All measured enzyme activities are extracellular ones.

The POC in the Darß-Zingst bodden chain, a shallow and tideless estuary, located south from the wind flat consists of 34 – 58 % of protein and of 21 – 37 % of carbohydrates (Schumann et al., 2001). Transferred to the findings in the wind flat, 0.2 – 0.6 mmol org. C cm^{-3} are proteins and 0.1 – 0.6 mmol org. C cm^{-3} were carbohydrates (Tab. 4.2).

The potential hydrolysis rate (Vmax) for the four aminopeptidases and four glycusidases are summed up to calculate the turnover time, assuming no interference between the enzymes. Also the compounds hydrolysed by the enzymes should be the only ones found in the organic material and should be evenly distributed.

Tab. 4.1: Organic carbon in the sediment and in biomass in the microbial mat in 2009. Organic carbon in sediment in mmol C cm^{-3} derived from organic content in % dry mass and assuming that 50 % of organic content are carbon. Organic carbon in biomass in µmol C cm^{-3} derived from factors for C-content per bacterial cell resp. per µg Chl *a*. Minima and maxima, median in brackets.

sediment		**biomass**	
Org. content (% dry mass)	0.6 – 5 (1)	Chl *a* (µg cm^{-3})	4 – 60 (30)
org. content (g cm^{-3})	10 – 370 (30)	Chl *a* (µmol C cm^{-3})	30 – 650 (220)
org. C (mmol cm^{-3})	0.5 – 15 (1)	bacteria (cells · 10^6 cm^{-3})	40 – 750 (230)
		bacteria (µmol C cm^{-3})	40 – 675 (210)
"degradeable free" org. C (mmol cm^{-3})	**0.3 – 4 (0.7)**	**org. C in biomass (mmol cm^{-3}) [% of total org. C)**	**0.3 – 0.6 (0.4) [10 – 60 (33)]**

The potential protein degradation is faster than the potential carbohydrate degradation and ranges within days (Tab 4.2). In March 2009, the protein degradation was fastest. The protein degradation was slightly slower in July 2009 and October 2009.

The turnover time of carbohydrates varied much more over the year compared to proteins. Only 10 – 20 days were needed in February, 2 – 3.5 months in June and in up to 3 months in September.

Tab. 4.2: Hypothetical carbohydrate and protein content in µmol C cm^{-3} in microbial mat in February, March, June, July, September and October 2009. Turnover time in d derived from corresponding Vmax-values in µmol C cm^{-3} for Glycusidases (Glyc.) and aminopeptidases (AP). Minima and maxima for protein and turnover, median for Vmax.

month	February	March	June	July	September	October
carbohydrate (µmol C cm^{-3})	120 – 210	X	150 – 260	X	330 – 580	X
protein (µmol C cm^{-3})	X	200 – 340	X	260 – 450	X	320 – 550
enzyme	Glyc.	AP	Glyc.	AP	Glyc.	AP
Vmax (µmol C cm^{-3} h^{-1})	0.38	9.8	0.1	9.1	0.28	6.5
Turnover (d)	10 – 20	0.8 – 1.4	60 – 110	1 – 2	50 – 90	2 – 5

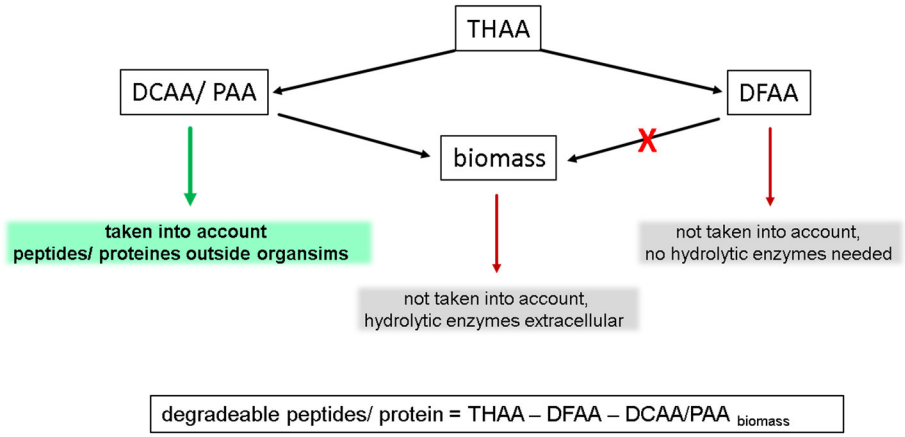


Fig. 4.2: Theoretical consideration about peptides and proteins which are degradeable by hydrolytic enzymes.

B. The protein turnover was evaluated in more detail with the results of HPLC analysis. Again, it has to be simplified which amount of the THAA are degradable. DFAA are not taken into account as no hydrolytic enzymes are needed to break them down. Furthermore, it has to be assessed how many peptides resp. proteins are bound in biomass. These biomass peptides and proteins (DCAA/ $PAA_{biomass}$) can also be neglected for turnover calculation under the consideration that only extracellular enzymes were measured (Fig. 4.2).

Not many data suitable do exist which feature the protein content of algae resp. bacteria. Especially for algae most of the literature deals with optimising growth and nutritional value of algae used in aquaculture. Also not all obtained data are reported in a way that they can be transferred to the findings in the wind flat. The chlorophyll *a* : protein ratio is used for estimating the protein bound in primary producers. The chlorophyll *a* : protein ratio increased from 1 : 14 (lag phase) to 1 : 160 (exponential phase) during growth of *Isochrysis sp.* (*Haptophyta*) (Valenzuale-Espinoza et al., 2001). The protein content of microbial mat primary producers is calculated with the 1 : 14 ratio as the primary producers should not be exponentially growing in the mat.

The cellular protein content of the bacteria was calculated with the percentual composition of the bacterial cell. Bacteria contain 10.4 – 53.5 fg $cell^{-1}$ (= 0.9 – 4.5 fmol C $cell^{-1}$) and 12.1 – 60.6 fg protein $cell^{-1}$ (= 0.6 – 2.7 fmol C $cell^{-1}$) (Simon and Azam, 1989). Hence bacterial cells consist to over 60 % of protein.

Primary producers' protein accounted for 14 – 26 µmol C cm^{-3} and the bacterial protein for 7 – 39 µmol C cm^{-3} in the wind flat microbial mat. The protein bound in biomass makes up 15 – 50 % of the TCAA/ PAA. Thus, 80 – 280 µmol C cm^{-3} are potentially degradable by aminopeptidases (Tab. 4.3). The four aminopeptidases need between 7 hours and 3 days to degrade all degradable DCAA/ PAA, when the potential hydrolysis rate (Vmax) for the four aminopeptidases are summed up (Tab. 4.4).

Tab. 4.3: Amino acids in in THAA, DFAA and DCAA/ PAA in µmol C cm^{-3} in the microbial mat in October 2009. Amino acids bound in biomass (DCAA/ $PAA_{biomass}$) derived from chlorophyll *a* and bacterial cell counts in µmol C cm^{-3} in October 2009. Minima and maxima values.

peptides/ proteins	AA (µmol C cm^{-3})	biomass	µmol C cm^{-3}	protein/peptides (µmol C cm^{-3})
THAA	190 - 420	Chl *a*	1 – 2	14 – 26
DFAA	20 – 140	Bacteria (total)	12 – 65	7 – 39
DCAA/ PAA	130 – 350		**sum DCAA/PAA $_{biomass}$**	**21 – 65**
degradeable peptides/ protein	**80 – 280**		**contributing % to DCAA/PAA**	**15 – 50**

Tab. 4.4: Total amino acids and individual amino acids Ala, Leu, Phe and Arg in different amino acids fraction in µmol C cm^{-3} in October 2009: total hydrolysable amina acids (THAA), dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA), particular amino acids (PAA). DCAA/PAA is dived into DCAA/PAA bound in biomass (DCAA/$PAA_{biomass}$) and DCAA/ PAA available for hydrolytic enzymes (degradeable DCAA/PAA). Vmax for Ala-, Leu-, Phe and Arg-aminopeptidase and for all four summed up (total) in µmol C $cm^{-3}h^{-1}$.Turnover time of degradeable DCAA/PAA in hours resp. days. Minima and maxima values.

amino acids	total	Ala	Leu	Phe	Arg
THAA (µmol C cm^{-3})	190 – 420	7 – 25	10 – 27	6 – 15	8 – 20
DFAA (µmol C cm^{-3})	20 – 140	0.8 – 8	0.8 - 12	0.5 – 4	
DCAA/PAA (µmol C cm^{-3})	130 – 350	3 – 19	5 – 24	7 – 14	8 – 20
DCAA/PAA $_{biomass}$ (µmol C cm^{-3})	21 – 65	0.7 – 5	1 – 6	0.9 – 4	2 – 7
degradeable DCAA/PAA (µmol C cm^{-3})	80 – 280	2 – 14	3 – 17	2 – 11	6 – 15
Vmax (µmol C cm^{-3} h^{-1})	3.7 – 12	0.9 – 1.8	1.2 – 5.4	0.9 – 7.2	0.24 – 0.54
turnover (h)	7 (hours) – 3 (days)	2 – 7	2 – 12	1 – 16	7 (hours – 3 (days)

The amount of the four amino acids (Ala, Leu, Phe and Arg) which is bound in biomass can also be calculated. 0.7 – 7 µmol C cm^{-3} of the individual amino acids of DCAA/PAA were bound in biomass. Hence, 2 – 17 µmol C cm^{-3} of the individual amino acids were degradable by the corresponding aminopeptidase. The aminopeptidases degrade their corresponding amino acid pool with different efficiency. Ala-, Leu- and Phe-aminopetidase just need 1 – 16 hours, whereas the Arg-aminopeptidase needs up to 3 days (Tab. 4.4).

4.0.3 Reliability of Ecoplate and Biolog substrate utilisation patterns

Biolog and Ecoplate plates with over 90 resp. 30 different carbon sources are a tool to analyse the substrate utilisation patterns of bacterial strains (Biolog) and communities (Ecoplate). They were originally designed to identify bacteria according to their substrate profile. This works more or less. 49 species of the genus *Pseudomonas* sensu lato from the culture collection of the University of Gent (Belgium) were analysed with the Biolog-System (224 strains in total). 24 species were identified correctly, others were almost correctly identified and a few completely wrong (Grimont et al., 1996).

The Biolog-System is more often used to evaluate the quality of carbon available to bacteria in their natural habitat, because it is more important to ecologists, what bacteria do than who they are. All studies agree more or less that the Biolog-System is a valuable tool to differ between communities. Unfortunately, the studies remain quite vague about the ecological implications of the substrate utilisation patterns.

The advantages of the Biolog-System are obvious: they are fast and easy to use and generate a large data set within no time. On the other side, the Biolog-System is a culture-based technique. Thus, it may be unrepresentative for the original community as faster growing organisms are favoured and a pseudo community may be formed due to selective enrichment (Konopka et al., 1998). Additionally, the physiological state of the bacteria influences the substrate utilisation kinetics and patterns (ibid.).

The substrate utilisation patterns and community composition (denaturing gradient gel electrophoresis (DGGE) - profiles) were analysed for microbial communities in activated sludge and the potato rhizosphere. Different numbers of bands were found in the different wells and the number of bands decreased the longer the incubation time was. More bacteria accumulated which were able to oxidise the respective C-source. However, there was one band which was present in all DGGE-profiles. Hence, at least one group of metabolic generalists should have been present in the samples (Smalla et al., 1998). Thus, the Biolog-System does not necessarily represent the real community composition but can give hints if the community is able to adapt to changing organic matter composition in their natural habitat.

The substrate utilisation patterns are influenced the most by the inoculum density and the incubation time. 77 % of variance within the substrate utilisation profile of one bacterial strain were due to different inoculum densities and incubation times (Kersters et al., 1997). 2 - 3 $\cdot$ 10^8 cells ml^{-1} are recommended by the manufacturer as inoculum density. Up to 17 substrates less were oxidised by one bacterial strain when inoculum densities of 10^4 cells ml^{-1} were applied instead of 10^8 cells ml^{-1} (Kersters et al., 1997). Cell densities of 10^8 cells ml^{-1} were difficult to reach for the wind flat microbial mat samples. Inoculum densities ranged between 0.3 - 1.1 $\cdot$ 10^6 cells ml^{-1} in microbial mat samples and 20 - 480 $\cdot$ 10^6 cells ml^{-1} for the bacterial strains. Thus, inoculum densities might

not have been high enough for a "correct" substrate utilisation pattern. Microbial mat samples were incubated longer time, keeping the possible artificial effects in mind. The inoculum densities for the bacterial strains were definitely high enough as the oxidation happened very fast (sometimes within seconds). All wells (also the control) within the plate were dark purple after 24 hours. Hence, higher inoculum densities might have caused no valuable results at all.

The incubation time should be not to long as all wells might give a positive oxidation results. Incubation time should be considered carefully when working with environmental samples. Only bacteria initially present and active and fast growing are considered at incubation times shorter than 24 hours. Furthermore, only C-sources which are metabolised quickly are recognised for the substrate utilisation pattern. More positive results are recorded at incubation times longer than 48 hours, but the differences between the samples vanish as well and the substrate utilisation patterns become more equal (Konopka et al., 1998).

Ecoplate community susbtrate utilisation patterns were incubated for 9 days. They were checked every day for new positive results. Only the positive results of the first two days were regarded as truly positive. Additional positive results after the first two days were noted separately (s. appendix for details). Important was the potential of the community to cope with (changing) organic material composition.

4.0.4 Inconsistent growth of bacteria isolated from environmental samples

Bacteria isolated from environmental samples grow much more inconsistent than laboratory "pets" like *Escherichia coli* featuring a reliable growth.

The analysed *Shewanella* sp - and *Pseudoalteromonas* sp -strain displayed always different growth rates in the experiments carried out. The inconsistent and ever changing growth of bacteria from environmental samples causes problems when making growth experiments and interpreting their result. Hence, one has to be aware of the different meanings of several growth parameters and choose wisely the ones interpreting.

The lag-phase is the time between inoculum and reaching the maximal division rate. The duration is determined by the conditions of the preparatory culture (medium, age of the culture etc.). Furthermore, bacteria might need some time to adapt to the new media. The exponential phase is characterised by a constant minimal generation time. In general, cell size and protein content should be the same for all cells. Cell numbers, protein content and dry mass increase proportionally to time. The growth rate is specific for the organism and depends only on the abiotic factors. Growth and cells might change during exponential growth phase in batch cultures as substrate concentrations decrease, cell density increases and metabolic products accumulate. Nevertheless, a specific growth rate can be calculated from the slope of the curve during exponential phase and it should always be

the same under the same conditions. Unfortunately, this is not true for bacteria isolated from environmental samples. The analysed *Shewanella* sp-strain displayed growth rates which differed up to 84 %. Growth rates can only range between 0 and 1. Thus, very different growth rates under the same conditions make it almost impossible to make proper interpretations without highly speculative consumptions why the organism grew slower or faster this time.

The gained growth rates of the *Shewanella* sp and *Pseudoalteromonas* sp strains are regarded relatively and not absolutly. They can give first hints and indications if the organisms grow faster than others but it is difficult to address which substrate conditions are optimal for the organism. Bacteria reach stationary growth phase when cell division and cell dead are equal. Decreasing substrate concentration is apart from high cell densities and accumulation of toxic metabolic products one reason. The population does not die immediately as long as the bacteria can gain the energy necessary by respiration of storage products and proteins (e.g. ribosoms). The capacity is the characterising parameter of the stationary growth phase. It indicates the maximum cell density of the culture under the prevailing conditions and depends on the conditions of the prepatory culture, the prevailing nutrient and substrate conditions and the culture conditions. Hence, the capacity explains more which substrates and nutrients are used more efficiently by the bacteria.

Furthermore, it is obligatory to know in which growth phase the bacteria are when analysing metabolic processes like enzyme activities or respiration. Extracellular enzymes may be suppressed during exponential growth and are just expressed when stationary growth is reached (Fuchs, 1999). The β-glucosidase activity of the *Shewanella* sp strain differed by 93 % on minimalmedium with starch resp. glucose in two experiments, measured at the same time under the same conditions. Without knowledge about the growth phase of the bacteria it would be impossible to interpret these results. The bacterial growth efficiency of two *Arthrobacter psychrolactophilis* strains from the eutrophic and humic flatland river Warnow ranged between 7 – 66 % and 17 – 84 % in three replicates under exactly the same conditions. (Warkentin, 2010). An over-interpretation might lead to wrong but fundamental important conclusions about the ecosystem functions, like if bacteria are respiring or producing within the ecosystem.

4.1 The wind flat microbial mat as habitat

The wind flat microbial mat as a special habitat will be discussed with the following hypothesis: The wind flat microbial mat presents a stable habitat compared to other microbial mats and coastal sediments. The wind flat microbial mat community is not as much affected by abiotic changes as other communities in microbial mats or sediments.

Microbial mats from tidal flats are flooded regularly. Hence, they face more mechanical stress than microbial mats from tideless regions. The examined wind flat microbial mat was most of the year non-flooded due to prevailing southern winds and moderate wind speeds. The mechanical impact on the wind flat microbial mat was rather lake-like. Thus, erosion of parts of the microbial mat by mechanical forces was minimal. These lake-like circumstances with less mechanical disturbances has influence on the distribution of the organisms in the wind flat.

Two dimensions of heterogeneity must be considered when looking at the distribution of organisms: time (seasonality or temporal variability) and space (patchiness or spatial heterogeneity). Nutrient availability and interactions between organisms can explain seasonal as well as spatial heterogeneity. Seasonal variability is obviously driven by oscillating abiotic factors, like temperature and photosynthetic active radiation (PAR). Spatial heterogeneity, especially in coastal areas, is driven by mechanic abiotic factors, like the structure of the sediment, erosion and wave forces.

Biomasses and species are heterogeneously distributed in sediments, thus patchiness is the "normal" phenomenon (Seuront and Spilmont, 2002). Spatial heterogeneity is often described on different scales: micro (cm – m), meso (m) and macro (m – km) (e.g. Plante et al., 1986, Saburova et al., 1995). Spatial heterogeneity is structured by sediment structure on the meso-scale. Biomasses accumulate behind these "little dunes" along transects of sand-waves on intertidal shores (Plante et al., 1985). The sediment structure has no influence on heterogeneity on the micro-scale. The sedimentary ripple marks are fast destroyed by waves and wind and hence, no biomasses accumulate at these structures (ibid.). Competition for nutrients and metabolic interactions are thought to be the main reason for this uneven biomass distribution on the micro-scale level (Saburova et al., 1995).

The spatial heterogeneity of biomasses was examined in May 2009 at 33 stations on the micro-scale level. None of the biomasses (chlorophyll *a*, pheophytin, bacteria) did correlate with each other. Bacteria were more heterogenously distributed than primary producers. Nevertheless, the biomasses within the wind flat microbial mat were homogeneously distributed due to absence of regularly mechanical forces caused by tides in contrast to tidal flats. The influence of regular mechanical forces on biomass fluctuations was observed in intertidal Australian mudflats. The upper sides were 30 resp. 50 % of the year non-flooded. They were nearly always flooded on the lower sides around the year. The chlorophyll *a* amount at the lower sides was not significantly different within one year.

Chlorophyll *a* increased threefold at one of the two upper sides (Cook et al., 2004a). Chlorophyll *a* from unconsolidated mats is up to five times more resuspended with increasing current velocity than from established mats (Lucas et al., 2000). There seems to be no general trend of the higher biomass accumulation at higher situated areas in intertidal flats (Guarini et al., 1998). However, the absence of mechanical forces like tides allows a stable biomass accumulation and the biomasses are spread more heterogeneously, like in the wind flat.

The distribution of biomasses within the depth of the microbial mat is triggered by physical and chemical gradients. The organisms accumulate in the horizon, where they find their favourable conditions (van Gemerden, 1993). Thus, stratified layers with different colours are formed because of the dominating organisms' characteristic colours. The wind flat microbial mats are different compared to the typical microbial mats found in tidal flats. The brownish diatom layer is found below the cyanobacteria layer. A stable layer of purple sulphur bacteria is not established (Fig. 4.1). However, the purple sulphur bacteria are also present in wind flat microbial mats. The relative photometric extinction for bacteriochlorophyll *a* (absorption maximum at 750 nm) was always between 0.02 – 0.04. Their abundance was just too low to form a visible purple layer.

The biomass variety within the upper first centimetre of the mat was examined. The examined layers were different in colour: green (0 – 3 mm) yellow-green (3 – 7 mm) and yellow/ single black spots (7 – 10 mm). The wind flat microbial mat was slightly thicker compared to other microbial mats. Furthermore, the different functional groups were not so clearly separated like in other microbial mats. The biomasses clearly accumulated in the upper 3 mm of the microbial mat, but the gradients were not so steep compared to other microbial mats (Tab 4.5).

The primary producers were mostly active (70 – 94 % active chlorophyll *a*) throughout the whole wind flat microbial mat. The green layer showed with 85 – 95 % the highest active chlorophyll *a* amount. In the deeper layers, the active chlorophyll *a* still accounted for 70 – 85 %. Microbial mats

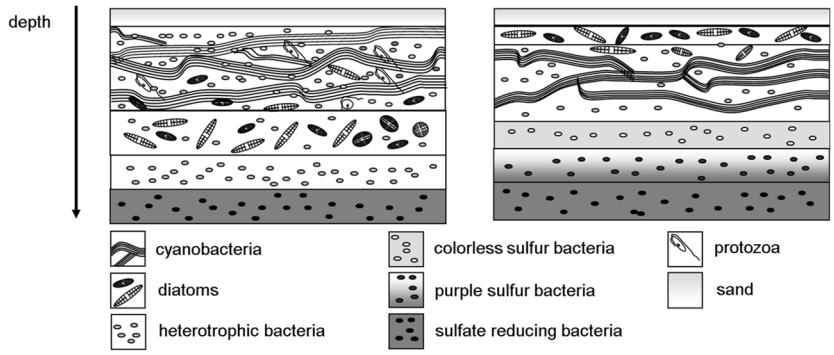


Fig. 4.3: Scheme of a typical wind flat microbial mat (left) and a typical tidal flat microbial mat (right). Source: Heyl et al., (2010)

Tab. 4.5: Depth profiles for primary producers in different microbial mats: depth in mm, colour of the layers and chlorophyll *a* in µg cm^{-3}

Mediterranean microbial mat, Ebro delta, Spain (Martinez-Alonso et al., 2004)			**Tidal flat microbial mat, Baja California (Stolz, 1990)**			**Wind flat microbial mat (this study)**		
depth (mm)	**colour**	**Chl *a* (µg cm^{-3})**	**depth (mm)**	**colour**	**Chl *a* (µg cm^{-3})**	**depth (mm)**	**colour**	**Chl *a* (µg cm^{-3})**
1 – 2	green	814	1	orange	39	0 – 3	green	50 – 66
2 – 4	brown	391	2.5	green	130	3 – 7	green - yellow	~ 22
4 – 5	pink	375	3.5	pink	72	7 – 10	yellow/ black spots	15 – 23
			4	salmon	99			

from tidal flats in the German Wadden Sea also showed a high active chlorophyll *a* fraction. No pheophytin was found in the upper 3 cm. Below 3 cm depth, it accounted only for 10 % (Billerbeck et al., 2007). Other microbial mats showed a clear depth gradient. The active chlorophyll *a* fraction decreased from 80 % in the upper 2 mm down to 30 % in 4 – 5 mm depth within a microbial mat of the Mediterranean Ebro Delta in Spain (Martinez-Alonso et al., 2004). Phytol and phytodiol as chlorophyll *a* degradation products were found in a hypersaline microbial mat. More than 80 % of the chlorophyll *a* was photodegraded due to high radiation (> 3000 µmol photons m^{-2} s^{-1}) in this microbial mat (Rontani and Volkman, 2005). The ATP-content (1400 ± 400 ng ATP g^{-1}) within the oxic layer (upper 2 mm) was up to five times higher and hence the organisms potentially five times more active than the organisms in the H_2S-layer of a hypersaline microbial mat (Ley et al., 2006). Also most of the heterotrophic bacteria were alive (70 – 90 %) and, hence, potentially active throughout the whole microbial mat. In contrast, large parts of the heterotrophic bacteria are dead or dormant in coastal sediments. Only 30 % of the total bacteria were active in Mediterranean sediments of the Adria, whereas 40 % were dormant and 30 % dead (Manini and Danovaro, 2006). Luna et al. (2002) found even up to 70 – 74 % dead and 20 – 29 % dormant bacteria in sediments of the Adria.It is not clear how many of these potentially active bacteria in wind flat microbial mat are really active. A large part of planctonic bacteria were alive (55 – 64 %), but only 9 – 14 % respired, 5 – 12 % showed esterase and only 3 – 6 % peptidase activity in the Darß-Zingst bodden chain (Schumann et al., 2003). Based on these findings, also only a minor part of the alive heterotrophic bacteria can be assumed to be probably active in the wind flat microbial mat.

Apart from the question, how active the wind flat microbial mat community is, the wind flat microbial mat provides favourable conditions for the heterotrophic bacteria community. The sediment in wind flat Bock is classified as medium sand which compared to finer sediments allows a deeper oxygen penetration (Woelfel et al., 2007). Like Woelfel et al. (2007) in 2004, no stable

purple layer was found and the black FeS-layer was present, but not always consolidated during 2009. These layers represent organisms depending on anoxic conditions: purple sulphur bacteria and sulphate reducing bacteria. As these organisms groups cannot establish permanently or in high abundances in the wind flat microbial mat, the community may find a good oxygen supply. A good oxygen supply may favour a stable, alive and potentially active microbial mat community.
Furthermore, microbial mats are a sticky mucoid matrix caused consiting of the extracellular polymeric substances (EPS) by the primary producers. The fixed CO_2 by microphytobenthos is to 62 – 77 % again released as EPS (Evrard et al., 2008). In August 2009, precipitation was with 32 mm low compared to May and November (60 resp. 85 mm) and temperature was about 19 °C in average during the week before sampling. The wind flat was non-flooded during sampling. All this causes rather "dry" circumstances in the wind flat. Many exopolymers have 99 % water content (Decho, 1994). Heterotrophic bacteria can more easily stay vital in this sticky matrix. Mucoid bacterial strains had a 35 % higher chance to survive desiccation compared with isogenic nonmucoid mutants in soils (Jass et al., 2002). EPS is not only consisting of a diverse easily degradable organic compounds pool produced by the primary producers, it entraps as well other surrounding polymers degradable by hydrolytic enzymes as well (Decho, 1994). Enzymes can also accumulate in EPS. It enables the community to stay active during "dry" periods and to regenerate faster. In a microbial mat in a salt marsh in northern Belize, the alkaline phosphatase activity remained constant over a period of 10 weeks desiccation (Sirova et al., 2006).

The biomasses within the wind flat microbial mat showed a typical seasonality with the highest chlorophyll *a* abundances during summer like found in other microbial mats (e.g. Pinckney et al., 1995, Karsten et al., 1998). Chlorophyll *a* increased twofold from March to August. Also in 2004, chlorophyll *a* increased by nearly 40 % from June to August up to 300 mg chl *a* m^{-2} within the wind flat microbial mat (Woelfel et al., 2007).
Pheophytin as "dead" biomass accounted for 4 – 30 % of the chlorophyll *a* amount. It was lowest during the summer months. Reasons for low pheophytin amounts might be good protection against photodegradation, active primary producers and/ or a high turnover of "dead" biomass by heterotrophic bacteria. The wind flat microbial mat was covered with sand year-around. Hence, photodegradation can be neglected. In contrast, > 80 % of the chlorophyll *a* was photodegraded due to high radiation in a hypersaline microbial mat (Rontani and Volkman, 2005). No pheopigments were found within a temperate Australian intertidal mudflat indicating a low grazing pressure (Cook et al. 2004a). A stable protist population, mostly ciliates, is found in wind flat microbial mats (Heyl et al. 2010). In 2009, an increasing number of nematodes were observed with naked eye during sample preparation. Thus, grazing pressure may now higher in the wind flat microbial mat than suspected before. Grazing pressure is next to bacterial or viral breakdown and autolytic cell lysis,

one reason for chlorophyll *a* degradation products found in sediments (Bianchi et al., 1988). However, protozoa like flagellates, amoebae, ciliates and some nematode-species, which were found within the wind flat microbial mat, are not able to feed on filamentous cyanobacteria and only burrowing meiofauna (gastropoda ad polychaetes) can destroy a microbial mat (Fenchel, 1998).
Information on spatial or temporal bacterial distribution in coastal sediments, flats and microbial mats is scarce. Bacterial abundances are recorded in the wind flat microbial mat since 2006. The bacterial abundances were very different through the years. Up to $12 \cdot 10^9$ cells cm^{-3} were found in the upper 3 mm (phototrophic layer) of the microbial mat in 2006 (Heyl, 2007). One year later, only $4 \cdot 10^9$ cells cm^{-3} were found in the phototrophic layer (Kern, 2008). Maximal $450 \cdot 10^6$ cells cm^{-3} were found in the microbial mat in 2009.
Bacterial abundances ranged between < 50 – 2000 cells $\cdot 10^6$ cm^{-3} in intertidal flats at the North Sea (Rusch et al., 2001, van Duyl and Kop, 1990). Thus, heterotrophic bacteria do clearly accumulate in the phototrophic layer of the wind flat microbial mat, but compared with other coastal sediments their abundance was not higher in microbial mats as it could have been expected. Most likely, the observed increasing abundances of nematodes together with a stable protist population within wind flat microbial mat probably had influence on the heterotrophic bacteria abundance. However, the chlorophyll *a* amount and the bacterial abundances within the microbial mat did, not reach as high amounts as in previous years (Tab. 4.6).
All in all, the wind flat with lacking regular mechanical disturbances by tides is a stable habitat in which microbial mats can establish permanently. The wind flat microbial mat does not feature steep gradients and a clear separation of the functional groups like in tidal microbial mats. In contrast to most of other sediments, the organisms are mostly alive and potentially active. Especially strictly anaerobic bacterial groups (purple sulphur bacteria and sulphate reducing bacteria) are not permanently present in the wind flat microbial mat. Hence, organisms may find a good oxygen supply which supports a stable, alive and vital microbial mat community. The EPS supports the microbial mat to overcome longer dry periods within the wind flat.
Nevertheless, purple sulphur bacteria are present within the microbial mat and are not lacking as it was thought before.
Although heterotrophic bacteria are clearly accumulating within the phototrophic layer of the microbial mat, their abundance is not higher as in other coastal sediments. The increasing abundance of nematodes and likely other protists as well feed on the bacteria.
In general, the microbial mat now seems to be in a degrading stage. The salt marsh community with *Puccinella maritima* (Hudson) Parlatore (common salt marsh grass) could established itself more and more over the years. Thus, the silting-up process within the wind flat is proceeding. The examined microbial mat might vanish from its actual location and colonise an until now mat-free area of the wind flat.

Tab. 4.6: Chlorophyll *a* in µg cm^{-3} and bacterial abundance in cells · 10^9 cm^{-3} within the wind flat microbial mat from 2006 – 2009.

Year	Months	Chl *a* (µg cm^{-3})	Bacteria (cells · 10^9 cm^{-3})	Source
2006	September – December	100 – 400	1.2 -12	Heyl (2007
2007	January – March	150 – 220	6 – 7	(Heyl (2007)
	September – December	40 – 400	0.8 – 4	Kern (2008)
2008	January – February	~ 60	0.6 – 2	Kern (2008)
	August – September	40 – 200	1.3 – 1.7	This study
2009	January – November	20 – 45	0.05 – 0.45	This study

4.2 Importance of hydrolytic enzymes to cover bacterial nutrition and to degrade organic material at the same time

DOM is with 750 Gt C the dominating form of organic carbon in aquatic oceans (Nagata, 2008). It ranges in the same order of magnitude with the amount of atmospheric CO_2 (750 Gt C) (ibid.). One of the biggest C-fluxes based on organic carbon in aquatic ecosystems is the one from detritus to microorganisms (Cole, 1999; Sala and Güde, 2004).

Hydrolytic enzyme activities give information about the degradation efficiency resp. ability of a community to degrade certain compounds of organic material. The meaning of hydrolytic enzyme activities within the wind flat microbial mat will be discussed with the following hypothesis:

Microbial mats are described to colonise extreme habitats (Van Gemerden, 1993). This may be true for the primary producers. The microbial mat itself cannot be regarded as an extreme habitat for heterotrophic bacteria. Heterotrophic bacteria find a sufficient substrate supply and benefit from the primary producers. The importance of aminopeptidases and glycosidases for the heterotrophic bacteria to gain substrates as alternative nitrogen sources and substrates for respiration will be addressed. Furthermore, the turnover times of proteins and carbohydrates (s. 4.0.2 for details) are evaluated with emphasis on the factors which influence the bioavailability and degradation efficiency (Fig. 4.4). First, the information gained by hydrolytic enzyme activities in respect of organic material degrading efficiency will be discussed. Later, the influence of the organic material composition and characteristics on bioavailabilty will be addressed.

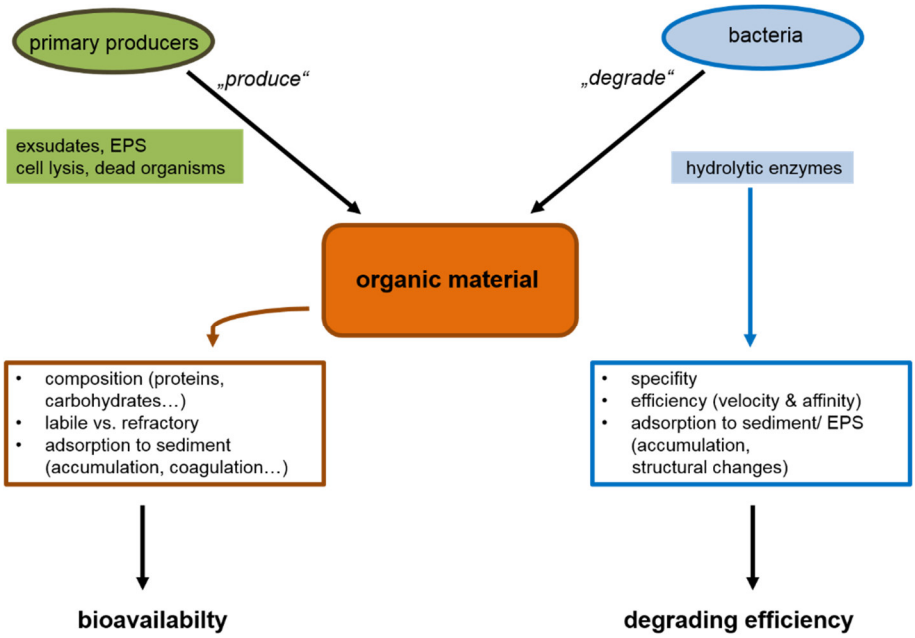


Fig. 4.4: Simplified scheme for production and degradation of organic material within the wind flat microbial mat with factors which influence bioavailabilty of organic material and degradation efficiency of hydrolytic enzymes.

Enzyme kinetics are rare in aquatic field studies. Only one study (Sirova et al., 2006) analysed hydrolytic enzyme activities in microbial mats so far. Therefore, hydrolytic enzyme activities in wind flat microbial mat are mainly compared to sediments and particle-attached (e.g. marine snow) hydrolases in aquatic environments.

The aminopeptidase Vmax-values showed no seasonal variation like the glycosidase Vmax-values and were on a high level throughout the year 2009. Aminopeptidase activities also exhibited the least seasonal variation in the Hudson River, but made up between 40 – 70 % of total hydrolytic activity (Taylor et al., 2003). The apparent aminopeptidase Vmax-values were one order of magnitude higher than the apparent glycosidase Vmax-values in the wind flat microbial mat. Hence, heterotrophic bacteria degraded preliminary proteins

Both Michaelis-Menten parameters, Vmax- and Km-values, decreased significantly from March to June 2009. If inorganic nitrogen or DFAA concentration rise, aminopeptidase Vmax-values will decrease (Chrost, 1991) as these nitrogen sources are more easily accessible. DFAA made up to 35 % of the THAA within the wind flat microbial mat in October 2009. The DCAA/ DFAA-ratio ranged from 1 – 13 in the wind flat microbial mat. Bacteria covered more of their C-demand by DFAA when DFAA amount was relatively high (DCAA/ DFAA ratio ~ 10) (Tab. 4.7). For example, DFAA covered 36 % of bacterial C-demand in the Gulf of Mexico in 1993, where DCAA/DFAA ratio was 8.5 (Kroer et al., 1994). One year later, the DCAA/DFAA ratio increased to 32. Then, DFAA covered only 2 – 7 % of bacterial C-demand, whereas DCAA covered 10 – 45 % of the bacterial C-demand (Kroer et al., 1994). Bacteria of the wind flat microbial mat community might preferably use DFAA to cover their C demand looking at the DCAA/ DFAA-ratios found (Tab 4.7).

Tab. 4.7: DCAA/ DFAA- ratio and % of bacterial C demand supplied by DFAA and DCAA in different areas. Source: Koer et al., 1994, modified.

Area	DCAA/ DFAA ratio	% bacterial C demand supplied by		Source
		DFAA	DCAA	
Gulf of Mexico	8.5	36	6	Jorgensen et al., 1994
Lake Constance	< 10	30 – 48	5 – 62	Rosenstock and Simon, 1993
Lake Constance	> 20	< 5	50 – 148	Rosenstock and Simon, 1993
Gulf of Mexico	32	2 – 7	10 – 45	Kroer et al., 1994
Wind flat microbial mat	1 – 13	main source	minor source	This study

Hence, are aminopeptidases of minor importance for the heterotrophic bacteria within the wind flat microbial mat? The constant high aminopeptidase activity throughout the year 2009 favours a general importance of these enzymes. Even more, the microphytobenthos is very efficient in NH_4- and NO_3- assimilation (Cook et al., 2004b). Thus, aminopeptidases are important for heterotrophic bacteria to access other nitrogen sources.

Nevertheless, the degrading efficiency and velocity was not very high, looking at the corresponding aminopeptidase activities to the actual substrate concentrations of the amino acids (Fig 4.5). Only the actual Ala-aminopeptidase activity was below the Km-value and 5 – 20 times lower than the Vmax-value (Fig. 4.5 A). All other aminopeptidase activities laid quite close to their Km-values, were even above them and hence "worked" slower when they came closer to the substrate saturation level. The actual activities were only 0.1 – 17 times lower than the Vmax-values. In contrast, the alkaline phosphatase activity was 1000 times lower than Vmax in an eutrophic lake (Chrost and Overbeck, 1987).

Furthermore, the aminopeptidases cleave more than just their name-giving amino acids from the peptides. Aminopeptidases are complex in their specificity and with which efficiency they hydrolyse peptides. They can cleave more than one amino acid (named P1 – P4 from their localisation next to the cleavage position) from the N-terminus of the peptide. The Leu-aminopeptidase (EC. 3.4.11.10) and Ala-aminopeptidase (EC. 3.4.11.2) are very unspecific aminopeptidases which cleave preferentially Leu and Ala but also more or less every other amino acid (s. Chap. 1.; Tab.1.1). Thus, it is likely, that the aminopeptidases worked above their saturation level as the prevailing substrate concentrations were of course higher when all amino acids are taken into account.

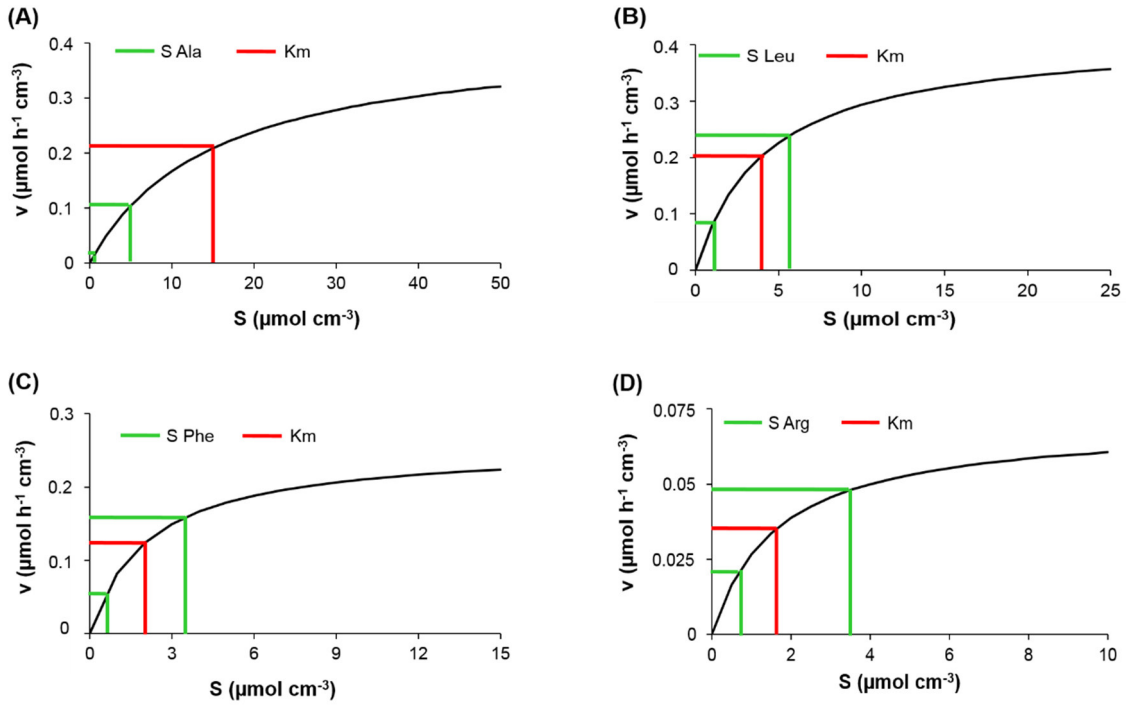


Fig. 4.5: Hydrolytic activity v in µmol h^{-1} cm^{-1} of Ala-aminopeptidas (A), Leu-aminopeptidase (B), Phe-aminopeptidaase (C) and Arg-aminopeptidase (D) within wind flat microbial mat in October 2009 (median). Indicated are Km (red line) and actual minimal and maximal hydrolytic activity under prevailing substrate concentration in µmol cm^{-3} (green line). Substrate concentration of Ala, Leu, Phe and Arg refers to DCAA/ PAA analysed via HPLC

There may be another, so far unregarded, enzyme alternative for the organisms to gain nitrogen. Ornithine made up up to 19 % in the DFAA within the microbial mat. Ornithine is the degraded form of Arginine and lacks two NH_x-groups at the side chain of the amino acid. The enzymes L-amino-acid-oxidase (EC 1.4.3.2) and D-amino-acid-oxidase (-dehydrogenase for bacteria) (EC 1.4.3.3) catalyse these reactions:

$$\text{D-amino acid} + H_2O + O_2 \rightarrow \alpha\text{-keto-acid} + H_2O_2 + \mathbf{NH_4}$$

$$\text{L-amino acid} + H_2O + O_2 \rightarrow \alpha\text{-keto-acid} + H_2O_2 + \mathbf{NH_3}$$

They are common enzymes and also cyanobactria possess them (Decho,1994). No amino acid uptake is needed with these enzymes, but NH_3 and NH_4 can be assimilated directly by the organisms. So far, the meaning and influence of these oxidases for N-cycling is not completely understood. They are activated when N is limited (Decho,1994). Organisms might prefer to cleave NH_3-residues directly from the amino acid, instead of amino acid hydrolysis and assimilation. Thus, they may be an effective alternative to cover the nitrogen demand of the organisms.

The glycosidic activities displayed a more complex pattern within the microbial mat. Hydrolytic activities reflect indirectly the prevailing substrate composition (e.g. Boetius and Lochte, 1994). α- and β-glucosidase activity made up 70 % of five measured glycosidic activities in an estuarine

microphytobenthos biofilm (Thornton et al., 2010). α- and β-glucosidase activities represented 65 – 80 % of the four examined glycosidic activities within the wind flat microbial mat in 2009 and 60 – 90 % in 2006/ 07 (Heyl, 2007). Hence, the degradeable carbohydrate pool in the wind flat microbial mat as well as in most coastal sediments may mainly consist of glucose. The relative cellobiodase activity was ten times higher in Finish lake sediments with 70 % conifers (delivering cellulose to the sea) in the catchement area than in lake sediments with only 5 % conifers (Hakulinen et al., 2005). If the activity of one enzyme increases this is linked with an increase of the corresponding enzyme substrate. Specific β-glycosidase and N-Acetylglucosaminidase activity increased 10 – 20 fold when cellulose and chitin were added to benthic microbial assemblages from the Arctic continental slope (Boetius and Lochte, 1996).

α- and β-glucosidase Vmax-values were on the same level in February and June 2009 in wind flat microbial mat. However, β-glucosidase Vmax-values were about four times higher than the α-glucosidase Vmax values in September 2009. Thus, more compounds with β-glycosidic bonds than α-glycosidic bonds were likely present in the wind flat microbial mat. The cellobiodase Vmax-values were always the lowest out of the 4 glycosidases. Hence, the disaccharide cellobiose as a relative "high-molecular" compound was likely only present at minor concentrations in the wind flat microbial mat.

The Km-values of the glycosidases were in the upper range compared with findings in other aquatic or terrestrial regions (Tab. 4.8). α- and β-glucosidase Km-values were significantly higher in a mesocosm consisting of phytoplankton cultures than in an unaltered seawater mesocosm. Organic material consisted mainly of low molecular weight compounds released by phytoplankton in the phytoplankton mesocosm. The bacteria in this mesocosm did not need to have a high affinity for the corresponding substrates as they were present in excess (Azua et al., 2003). Additionally, bacteria attached to aggregates expressed hydrolytic enzymes with higher Km-values as bacteria in ambient waters. The Km-value was higher due to substrate accumulation and concentration in aggregates (ibid.).

The Km-values for glycosidases are comparable with the ones found in the phytoplankton mesocosm and support the hypothesis of a high carbohydrate amount degradeable by α- and β-glucosidase within the wind flat microbial mat. Furthermore, the carbohydrates can be considered to be of low molecular weight and easily utilizable by bacteria.

So far, hydrolytic enzymes seem to be of minor importance for the bacterial nutrition and the degradation of organic material: They showed a low affinity for their corresponding substrates and worked quite close to the substrate saturation level or what is likely as well even above it.

Tab. 4.8: Km-values in µmol l^{-1} for α-glucosidase (α-gluc.), β-glucosidase (β-gluc.), cellobiodase (cellob.) and N-Acetylglucosaminidase (NAC) in different areas. HM = high molecular, LM = low molecular, OM = organic material, amb. = ambient.

Area		Km (µmol l^{-1})				Source
		α-Gluc.	β-Gluc.	cellob.	NAC	
Mesocosm unaltered sea water	HM OM aggregates	4 – 19	1 – 64			Azua et al., 2003
	HM OM amb. water	0.2 – 3	0.03 – 6			
Mesocosm phytoplankton cultures	LM OM aggregates	50 – 330	1 – 75			
	LM OM amb. water	4 – 45	12 – 35			
Grassland soil (Ireland)	low N-load		26	2		Marx et al., 2005
	high N-load		21	13	35	
Adria (Croatia) – mesocosm		0.06 – 1	0.04 – 2			Agis et al., 1998
Wind flat microbial mat	Feb. 2009	10 – 55	20 – 30	20 – 30	21 – 24	this study
	Jun. 2009	30	35	110	16	
	Sep. 2009	10 – 20	40 – 240	3 – 45	5 – 40	

However, the question remains why the bacteria within the wind flat microbial mat expressed such a very high hydrolytic activity when they could theoretically cover most of their nutrient demand by assimilation of dissolved free compounds. The Vmax-values of the Leu-aminopeptidase, α- and β-glucosidase were 3 – 13 times higher than in a mesocosm with low molecular weight organic material (Azua et al., 2003).

Looking at the amount, composition and bioavailabilty of the organic material, the organic material somehow "determines and influences" its degrading velocity. The organic material composition was analysed resp. estimated with the help of literature data to balance how effective hydrolytic enzymes degrade the compounds present in the wind flat microbial mat (s. 4.0.2.2). The amount of protein and carbohydrates within the wind flat microbial mat was higher than at other stations (Tab. 4.9). It was up 2000-times higher than in the water of the eutrophic Darß-Zingst bodden chain, an inner coastal lagoon south of the wind flat. DFAA amount was also up to 200 times higher than in deep sea sediments (- 3500m) in Arctic continental slope (Boetius and Damm, 1998). The protein and carbohydrate was in the same range with a sediment in Gulf of Trieste (microphytobenthos in 20 m depth). Protein amount was only around 4 times lower and carbohydrate amount only 5 – 12 times lower than in wind flat microbial mat (Welker et al., 2002).

Tab.4.9: Protein and carbohydrates at different stations. Bulk protein/ carbohydrate referes to total protein/carbohydrate (originally expressed as BSA- resp. glucose-equivalents); except this study = without protein/ carbohydrate bound in biomass and Schumann et al. (2001) = reference value is POC. Values are in italics, if proteins/ carbohydrates were analysed as DFAA/DFCHO and DCAA/DCCHO. All original literature values were converted into µmol C cm^{-3}. Sediment* = sediment with microphytobenthos

Station	type	Protein (µmol C cm^{-3})		Carbohydrate (µmol cm^{-3})		Source
		DFAA	DCAA	DFCHO	DCCHO	
Darß – Zingst bodden chain	water	0.1 – 0.9		0.1 – 0.6		Schumann et al, 2001
Darß – Zingst bodden chain	water	*0.03*	*0.3*	*0.07*	*2*	Görs et al., 2007
Arctic continental slope (East Russia)	sediment	5 – 40				Boetius and Damm, 1998
		0.1 – 3.5				
Mediterranen Sea (Apulia)	sediment	8 – 14		50 – 80		Dell'Anno et al., 2002
Northern Adriatic Sea (Gulf of Trieste)	sediment*	60 – 125		25 – 50		Welker et al., 2002
Wind flat microbial mat	sediment	200 – 500		120 – 580		this study
		20 – 140	*130 – 350*			

Hence, the organic material found in the wind flat microbial mat was at the upper range compared with other stations. The protein and carbohydrate content was higher than in most of the other regions (Tab. 4.9).

The rather slow turnover of peptides and carbohydrates within the wind flat microbial mat are striking, when compared with organic material turnover times at other stations (Tab. 4.10). Turnover times for dissolved combined and particulate amino acids and carbohydrates were definitively longer than at other stations. Proteins were more rapidly degraded at other stations. Sometimes it took only a few hours, like in the Sea of Azov (Agatova et al., 2008). The protein degradation was also 12 – 22 times faster than the carbohydrate degradation within the wind flat microbial mat. It would take up to nearly 4 months to degrade all carbohydrates within the wind flat microbial mat.

Finally, the bioavailabilty of the organic material may explain the slow turnover of organic material. Bacteria, their extracellular hydrolytic enzymes and the organic material are most likely attached to particles. This can lead to accumulation of organisms, organic material and activites at various hotspots (Azua et al., 2003). Furthermore, the EPS within microbial mats entraps, stores and accumulates organic material as well. Attachment to particles may lower bioavailability of organic material and potential activity of organisms at the same time (Quiquampoix et al., 2002)

Tab. 4.10 Turnover times (d) for organic material at different stations. Different types of organic material were analysed/ used for turnover calculation in the different studies: without in biomass bound material for the wind flat microbial mat in this study

Station	Type of organic material	Turnover time (d)		Scource
Indian Ocean	POM & DOM	8 (POM)	26 (DOM)	Misic et al., (2006)
Sea of Azov (Russia)	PCAA	1.7 – 3.9 (hours)		Agatova et al., (2008)
Southern California Bight	PCAA on marine snow	0.2 – 2.1		Smith et al., (1992)
Wind flat microbial mat	DCAA /PCAA & DCCHO/ PCCHO	0.8 – 5 (peptides)	10 – 110 (carbohydrates)	This study

In general, viscosity and diffusion determine the bacterial (micro-)environment. Bacteria cannot move without a flagellum because of water viscosity (Jorgensen, 1995). Diffusion is the fastest way of transportation and faster as the bacterial locomotion (ibid.). Heterotrophic bacteria have a border of 10 µm around their cell, in which they obtain a positive return of released substrates by extracellular hydrolytic enzymes relative to the energy investment of expressing, building and releasing these enzymes (Schulz and Jorgensen, 2001). Theoretical every substrate should meet every enzyme within 1 second within the bacterial cell due to the Brownian movement (ibid.). Hence, the bacteria and their "working" conditions should not influence negatively the organic material degradation efficiency by hydrolytic enzymes.

However, external circumstances in the wind flat microbial mat are different: Bacteria attached to particles are surrounded by a capsule-like matrix (Heissenberger et al., 1996). This can lead to an enzyme accumulation. The capsule can also be an additional diffusive boundary layer (Agis et al., 1998). Thus, bound enzymes are often not able to hydrolyse the substrate quite "close" to their cell as it is not recognized because of the diffusive boundary layer (Wetzel, 1991).

The substrates as well can be excluded from degradation by bacteria. Polymers are hardly soluble in water (Fuchs, 1999). EPS tends to coagulate and aggregate (Bhaskar et al., 2005) which lowers bioavailability. For instance, liposomes as "flagellate fecal pellets" form in aquatic environments (Nagata and Kirchman, 1992). Molecules and compounds can be entrapped in the liposome capsule during liposome formation (New, 1990). Protein degradation lasted three times longer when protein was attached to liposomes, no matter if encapsulated within the liposome or attached at the outer liposome membrane (Borch and Kirchman, 1999). Heterotrophic nanoflagellates were found in relatively high abundances (70 – 380 individua cm^{-3}) within the wind flat microbial mat (Heyl et al., 2010). Liposomes entrapping proteins, carbohydrates and other organic material are likely to be

present within wind flat microbial mat and lowering bioavailabilty of substrates resp. degradation efficiency by hydrolytic enzymes.

Despite of liposomes, every attachment to particles lowers bioavailabilty. Especially proteins have a high affinity to attach to surfaces. Proteins are ellipsoids in their tertiary structure. Less surface of the protein is free when attached to a surface. The active centre of the enzymes may be blocked by surface attachment, for instance (Quixquampoix et al.,2002).

Some parts of DOC can be resistant for up to 6000 years (Willams and Druffel, 1987) because of such small but significant and sometimes even irreversible changes within the molecular structure of attached substances.

The DOM in aquatic ecosystems can be divided into three groups: labile with a low molecular weight (LMW) and a turnover time of less than hours up to days; semilabile with a high molecular weight (HMW) and a turnover time of months to years; and refractory with a LMW and a turnover time of up 1000 years (Fig. 4.6; Nakata, 2008). Less than 1 % of DOM in aquatic ecosystems is labile and fast degradable by hydrolytic enzymes (ibid.). The DOC released by a bloom of diatoms consisted to 60 % of substances with < 900 g mol^{-1} and less than 20 % with > 2500 g mol^{-1}. Heterotrophic bacteria assimilated and respired 39 – 99 % of the released DOC. Low molecular weight substances did not accumulate (Jensen, 1983). Hence, an accumulation of more refractory organic material is a normal phenomenon.

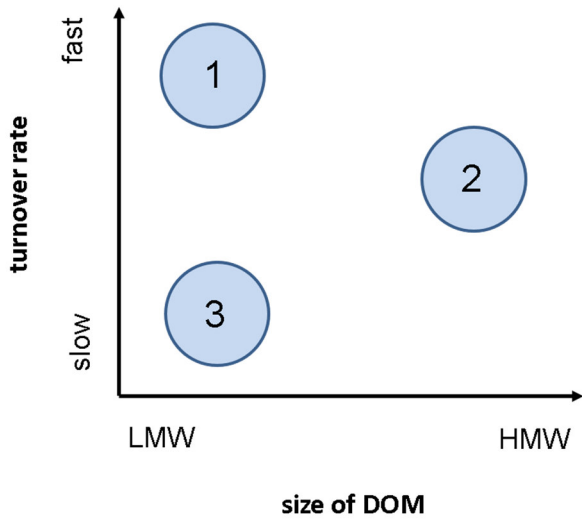


Fig. 4.6: DOM turnover rates sorted after DOM size (low molecular weight – LMW to high molecular weight –HMW) and turnover rate by hydrolytic enzymes: 1 – labile material, like amino acids and free sugars; 2 – semilabile material, like organic polymers; 3 – refractory material not recognised by enzymes as substrate due to structural modifications (adsorption of minerals, glycolysation etc.). Source: Nakata, 2008.

All in all, the actual organic material accumulation within the wind flat by the microbial mat is a natural process, together with the observed ongoing silting-up process (as described in 4.1).
The examined hydrolytic enzymes have a low affinity for their corresponding substrate and their activities are close and/ or even above the saturation level. Thus, the organic material turnover is very slow. Nevertheless, this does not necessary prove the unimportance of hydrolytic enzymes for the organic material turnover. Some amount of the organic material is likely excluded from the bioavailable organic material pool by attachment to sediment particles.
From this point of view, the microbial mat heterotrophic bacteria use other strategies with more efficiency to cover their nutrient and substrate demand. They likely predominantly assimilate free and dissolved organic material with low molecular weight. So far unregarded enzymes, like the amino-acid-oxidases, might be an efficient alternative to cover the nitrogen demand of the organisms.

4.3 Potential of microbial mats to cope with changing organic load

There is an ongoing discussion how microbial communities react and adopt themselves to changing organic load. Two theories are discussed within the scientific community: 1. Community composition changes because bacteria become more abundant which are able to utilise the changed organic material composition. 2. All organisms are able to adopt their metabolism to the new conditions (Sala and Güde, 2004). Both theories have good arguments on their site. On the one hand, microbial metabolism is known for its high ability to adopt to changing substrate concentrations (Poremba and Hoppe, 1995). On the other hand, some bacteria are always abundant under certain organic and nutrient loads in the aquatic environment (e.g. Cottrell and Kirchmann, 2000).
The ability to degrade certain organic compounds by bacteria isolated from the wind flat microbial mat was analysed in this study. Emphasis laid on the inducibilty of glycolytic enzymes by mono-, di- and polysaccharids and its implementations for degradation of high molecular weight carbohydrates, like starch, cellulose or chitin. The following hypothesis will be discussed: Heterotrophic bacteria find a sufficient supply of easily accessible substrates within the microbial mat. They do not feature a great metabolic flexibility as the wind flat microbial mat is not characterised by steep gradients and ever changing abiotic parameters compared with other microbial mats and coastal sediments. Especially their ability to degrade high molecular compounds is limited.
The microbial mat community displayed different substrate utilisation patterns in different months. Primarily carbohydrates were oxidised in May, amino acids in August and as many carbohydrates as amino acids in October. The least C-sources were oxidised in August within the microbial mat and primarily amino acids were respired by the community. It might be an indication for a different

substrate composition as well as a different community composition within the microbial mat. The bacteria within a manure compost also displayed different substrate utilistation patterns during the compost process. Especially simple carbohydrates (Fructose and Galactose) were oxidised in the young compost, whereas polymers and a number of carbohydrates were oxidised to a greater degree in older compost (Insam et al., 1996).

However, 11 additional C-sources were oxidised after 48 h incubation in August 2009. Hence, the microbial mat community can adapt rapidly to changing substrate composition. Especially polymers were always respired very fast year-round. The microbial metabolism is able to adapt fast to changing environmental conditions (Poremba and Hoppe, 1995). Bacteria can build energy demanding and exceptional enzymes, but only if no limitations like shortage of inorganic nutrients are present (Taylor et al., 2003).

Nevertheless, the microbial mat community seems to be special regarding its ability to oxidise complex compounds despite the dominating presence of easily accessible and low molecular weight compounds in the microbial mat. In contrast, heterotrophic bacteria only oxidised simple sugars in soils amended with glucose and poorly oxidised amino acids and polymers (Schutter and Dick, 2001).

Nearly all isolated bacteria from the wind flat microbial mat belong to the γ-Proteobacteria. One strain was identified as *Bacillus baekyrungensis*, a $Gram^+$ -bacterium with low GC-content. The genus *Bacillus* covers many bacteria representing different kinds of physiology, ecology, genetics and habitats (Slepecky and Hemphill, 2006). Unfortunately, little is known about the physiological and metabolic characteristics of *B. baekyrungensis.* The isolates of the γ-Proteobacteria represent the genera *Pseudomonas*, *Vibrio, Shewanella* and *Pseudoalteromonas*. γ-Proteobacteria are widely spread in oligotrophic oceans and coastal waters (Rappé et al., 1997). Thus, they can be seen as typical wind flat bacteria.

Pseudomonas is known for its genetic and metabolic adaptability with simple nutritional requirements and able to metabolize an extensive number of substrates. It only requires aerobic conditions. It is ubiquitous and any habitat with temperatures of 4 – 42 °C, pH 4 -8 and containing simple or complex organic compounds is a potential habitat for *Pseudomonas* (Moore et al., 2006).

The genus *Vibrio* is divided into "medical" (e.g. *V. cholerae*) and marine species. *Vibrio* can only use a limited amount of organic compounds as energy sources. Cellulose, formate, dicarboxylic acids with 6 – 10 C-atoms, L-isoleucine, L-valine, L-lysine, L-tryptophane. Purines, pyrmidines and n-hexadecane cannot be used at all (Farmer III and Hickman-Brenner, 2006).

The in more detail analysed *Shewanella*- and *Pseudoalteromonas*-strains can be regarded as typical representatives for the wind flat microbial mat. Both are quite different regarding their metabolic capacities but both reflect the conditions within the wind flat microbial mat. *Shewanella* can be considered as metabolic extremist, whereas *Pseudoalteromonas* is rather a metabolic generalist.

Shewanella is an interface organism and features a "gradient lifestyle". It is generally found in habitats with rapidly changing redox-conditions, utilising a variety of electron-acceptors and alternating oxic/ anoxic conditions. Its common metabolic carbon sources are lactate, formate and amino acids. It cannot use complex carbon sources (Nealson and Scott, 2006). *Pseudoalteromonas* is an obligate marine organism and so far never has been found in terrestrial habitats as it needs NaCl for growth. It is widespread in any kind of marine habitat and can utilise a variety of organic C-compounds ranging from carbohydrates, monocarboxylic acids, amino acids and D-glucose (Mikailov et al., 2006).

The substrate utilisation profiles reflected the general characteristics of the wind flat microbial mat isolates. *Shewanella sp*. and *Shewanella baltica* oxidised the most C-sources due to their metabolic adaptation to ever changing environmental conditions. They utilised many amino acids and carbohydrates. Even complex carbohydrates like dextrine (starch degradation product) were oxidised, although *Shewanella* is said not to utilise rather complex C-sources (Nealson and Scott, 2006). *Pseudomonas* and *Pseudoalteromonas* strains showed a rather narrow metabolic spectrum in contrast to the *Shewanella* strains. They do not feature a "gradient-lifestyle" and hence do not have to be adapted that much to ever changing environmental parameters. Nevertheless, some *Pseudomonas* species are able to utilise up to 200 different C-sources (Fuchs, 1999).

In general, two results of the substrate profiles are striking. The isolates oxidised more carbohydrates than amino acids in contrast to the community substrate profiles. It is quite likely to be an artifical effect. The proteolytic activity is often higher in environmental samples than in culture (Martinez et al., 1996). A good nitrogen supply, like in peptone containing media, leads to higher glycolytic activity (Misic and Harriague, 2007). Hence, a good nitrogen supply within the medium, might affect the substrate utilisation just as the hydrolytic activities of the bacteria. More important, all isolates were able to oxidise nearly all polymers within the Biolog plates. Hence, wind flat microbial mat bacteria are able to utilise rather complex C-scources and are not only adapted to simple C-scources provided by the primary producers.

Also N-Acetylglucosamine and cellobiose, components of polysaccharids chitin and cellulose were oxidised by nearly all isolates. Large parts of organic material consist of cellulose and chitin. Cellulose is the main component of higher plants and thought to be the most abundant organic polymer on earth (Klemm et al., 2005). Chitin is after cellulose the second most abundant polysaccharide on earth (Taranathan and Kittur, 2003). Hence, oxidisation of their small subunits N-Acetylglucosamine and cellobiose is not surprising as it is the last step of organic material degradation.

Polysaccharid degradation into accessible and available subunits by bacteria is a complex progress. Hydrolytic enzymes are the last step within break-down of these polymers. The four analysed wind flat microbial mat isolates of the genera *Pseudoalteromonas* and *Shewanella* all displayed a

constitutive α-glycolytic- and N-Acetylglucosaminic-activity. Two showed β-glycolytic and cellobiodase activity and for one strain β-glucosidase activity was induced.

Glycogen is the bacterial storage carbohydrate consisting of α-1,4-linked glucose branches. These branches are linked to each other via α-1,6-linkages every 8th to 12th glucose molecule. Thus α-glycolytic activities of all analysed strains is not surprising.

However, cellobiodase, β-glucosidase and N-Acetylglucosaminidase are the final steps in cellulose and chitin degradation. It cannot be said, if these wind flat microbial mat bacteria are really able to degrade these complex compounds. "Truly" cellulytic and chitolytic bacteria seem to be scare in aquatic environments. Up to 90 % of ~ 1000 bacterial strains from a sandy beach at the Southern Baltic Sea (Sopot, Poland) were able to degrade starch, whereas only up to 9 % could degrade chitin and nearly no strain was able to degrade cellulose (Podgorska and Mudryk, 2003).

Bacteria display different strategies and can express a broad range of different enzymes to degrade the rather "simple but diverse" molecule cellulose: chains of 100 – 10,000 β-1,4-linked glucose molecules. The disaccharids cellobiose are rotated 180° to each other and the chains are arranged parallel to each other linked via H-bonds (Fuchs, 1999). The cellulosomal system for degrading cellulose is the most complex one. It is an extracellular macromolecular "machine" consisting of different subunits: 1. cellulose binding domain which binds to cellulose 2. catalytic domains which are responsible for hydrolysis. 3. Cohesin- and dockerin-protein-domains which integrate and arrange the respective enzymatic subunits within the cellulosom (Bayer et al., 2006).

One organism can express many different enzymes involved in cellulose degradation. For instance, *Clostridium thermocellum*, which "feeds" only on cellulose, expresses many different endo- and exo-β-glucanases and 5 xylanases, carbohydrate esterase, lichenase, mannase and chitinase. The latter hydrolases cleave interfering compounds like lignin from cellulose (Bayer et al., 2006).

Saccharophagus degradans posesses 13 different endoglucanases, 5 different β-glucosidases (normal are 1 – 2) and furthermore also one phosphorolytic cellobiase (Zhang et al., 2011). Glucose-1-phosphate is the product of phosphorylytic cellobiase activity, which can easily be transferred into Glucose-6-phosphate, an intermediate product during glycogen-synthesis. Thus, phosphorylyse is an energy conserving process as it lowers the amount of ATP necessary for maintenance processes of the cell.

Chitin has a homologous structure like cellulose, except for its components which are N-Acetylglucosamine. The chitin degradation is not as complex as the cellulose degradation. Chitinases cleave chitin into water soluble N-Acetylglucosomine oligomers and dimers. The dimers are cleaved into monomers by β-N-Acetylhexosaminidases (Beier and Bertilsson, 2013). Some bacteria also express a chitin-binding-protein which transfers the christalline chitin β-folding into the more easily degradeable α-folding (ibid.).

A so called “interspecific crossfeeding” of “satellite microorganisms” is more likely widespread among bacteria as only a few seem to be able to degrade cellulose and chitin directly. If cellulytic bacteria degrade cellulose to smaller subunits like cellobiose or glucose, “satellite microorganism” may assimilate these compounds as well (Bayer et al., 2006). The hydrolysis products cellobiose and glucose can be further utilised by „satellite microorganisms” which are not capable to degrade cellulose alone (Bayer et al., 2006). 0.1 – 5.8 % bacteria of a variety of aquatic habitats can be regarded as potential chitinolytic bacteria as chitinase genes were found in their metagenome (Beier and Bertilsson, 2013). Only 0 – 1.9 % express a chitinolytic activity, but 4 – 40 % assimilate the degradation products (N-Acetylglucosamine as monomer or dimer) (ibid.). Lateral gene transfer is one reason why more bacteria possess chitinase genes than bacteria expressing chitinolytic activity (ibid.).

The influence of different carbohydrates on growth and hydrolytic enzyme activity (α- and β-glucosidase) was analysed for one *Shewanella* sp - and one *Pseudoalteromonas* sp - strain. Bacterial growth at different substrate conditions can give insights how bacteria deal with low substrate concentrations within marine environments (Kirchman et al., 2003). However, low substrate concentrations are not quite likely within the wind flat microbial mat. It is more a question of bioavailabilty (s. 4.2 for details) which might lead to low substrate concentrations as well.

Bacteria grow relatively slow in aquatic environment with generation times in the order of days which are much longer than the ones for bacteria growing in the laboratory with generation times in the order of 30 minutes (Kirchman, 2012). Growth rates of 0.2 – 0.9 d^{-1} were found for bacterioplankton at different stations in the Gulf of Mexico and the Mississippi River plume (Jochem et al., 2004). In the laboratory, higher growth rates are normally found with strains from aquatic environments. Growth rates of 0.15 h^{-1} up to 0.8 h^{-1} were found for marine bacteria (Kirchman et al., 2003, Law and Button, 1977). The growth rates of *Shewanella* sp and *Pseudoalteromonas* sp strains from the wind flat microbial were around 0.15 h^{-1} and 0.07 h^{-1} under most condition. Thus, they grew slower than other bacteria isolates. The relatively slow growth reflects the conditions within the microbial mat. As the bacteria seem to grow relatively slow, they can be regarded as typical K-strategists, which are adapted to stable and not ever-changing environmental conditions.

Shewanella sp was the only wind flat microbial mat bacterium able to grow on minimalmedium with NH_4^+ as inorganic nitrogen scource. All other strains only grew on peptone containing media and hence needed with amino acids in the medium an organic nitrogen source to grow. Bacteria tend to grow faster when they can assimilate amino acids directly. *Vibrio harvey* grew up to 2 times faster when amino acids instead of NH_4^+ were added to the medium (Kirchman, 2003). A 25 times lower glucose concentration was necessary for positive growth of a marine *Corynebacterium* when Arginine was also added to the medium, instead when *Corynebacterium* had to grow on glucose

alone (Law and Button, 1977). However, the transport costs of using several amino acids might exceed the energetic advantage of avoiding amino acid synthesis, which is necessary for cells growing on NH_4^+ as nitrogen source (Kirchman et al., 2003).

The *Shewanella* sp strain was able to grow with amino acids and NH_4^+ as nitrogen source. Amino acids tended to be its preferable nitrogen source as the capacities reached on these media were 10 to 20 times higher than on the ones with NH_4^+ and an extra carbon source. It also reflects the enormous importance of amino acids in aquatic environments as they fulfil the double role of nitrogen and carbon source.

Also the α- and β-glucosidase activities of the *Shewanella* sp and *Pseudoalteromonas* sp strain, recorded during the 10 days experiment, support the until now known general characteristics of these species. *Shewanella* sp, the "interface organism", showed no big difference in α- and β-glucosidase activity no matter which carbohydrate was added to the medium. Only the α-glucosidase activity was slightly higher when starch was added to the minimalmedium. The *Shewanella* genus is adapted to rapid changing environmental conditions. Thus, it seems to maintain some metabolic processes on a constant level to be able to react on a sudden substrate change.

In contrast, *Pseudoalteromonas* sp adjusted its α- and β-glucosidase activity much more to the prevailing substrate conditions. The induced β-glucosidase activity was between 20 to 270 times higher than the α-glucosidase activity, when cellobiose was added to the medium. On the other side, the α-glucosidase activity was up to 12 times higher than the β-glucosidase activity when starch was added to the medium. The *Pseudoalteromonas* genus is much more adapted to rather stable environmental conditions. It is able to adjust to changing substrate conditions, but in order to keep energy cost of these processes low it might have to deal with lower growth or other restrictions.

To sum up, the hypothesis has to be neglected. The microbial mat community is able to utilize complex C-compounds and is not only adapted to the easily accessible compounds provided by the primary producers. The bacterial isolates are typical for the wind flat microbial mat. In general, the *Shewanella* sp and *Pseudoalteromonas* sp strains are both K-strategists because of their slow growth. Nevertheless, both represent different "lifestyle" strategies which are typical for the microbial mat. These two at first contradictory "lifestyles" of adaption to interface/ gradient conditions and adaptation to stable/ slowly changing environmental conditions highlight the existence of different habitats on a small area within the microbial mat.

5. Summary and outlook

The microbial mat within the wind flat “Bock” at the southern Baltic Sea coast was analyzed in terms of its habitat characteristics, the importance of hydrolytic enzymes for the turnover of organic material and the ability of the heterotrophic bacteria community to adapt itself to changing organic material load.

The absence of regular mechanical disturbances by tides is likely the most important factor determining the habitat. The biomasses within the microbial mat are homogeneously distributed and the microbial mat can establish permanently within the wind flat. It does not feature steep gradients and a clear separation of the functional groups like in tidal microbial mats. Especially strictly anaerobic bacterial groups (purple sulphur bacteria and sulphate reducing bacteria) are not permanently present in high abundances in the wind flat microbial mat. Nevertheless, purple sulphur bacteria are present within the microbial mat and are not lacking as it was thought before.

As these anaerobic organism groups are not predominantly present within the in the wind flat microbial mat, a good oxygen supply might favour a stable, alive and potentially active microbial mat community. Indeed, the primary producers are mostly active in contrast to normal coastal sediment. Also the major part of the heterotrophic bacteria is alive and potentially active. They do clearly accumulate in the phototrophic layer of the wind flat microbial mat, but compared with other coastal sediments their abundance was not higher in microbial mats as it could have been expected. Most likely, the observed increasing abundances of nematodes together with a stable protist population within wind flat microbial mat probably has influence on the heterotrophic bacteria abundance.

The microbial mat is a good habitat for heterotrophic bacteria which benefit from the exudates of the primary producers. The primary producers most likely release exudates which are free and dissolved compounds with low molecular weight. These compounds are easy to assimilate and the heterotrophic bacteria can mainly cover their substrate demand by these compounds. So far unregarded enzymes, like the amino-acid-oxidases, might be another efficient alternative to cover the nitrogen demand of the organisms. Nevertheless, the heterotrophic bacteria are able to adopt themselves to changing organic material compositions, although they rely mostly on easily accessible substrates. When complex substrates with high molecular weight are the only C-source present, they are used very fast. The bacterial isolates are typical for the wind flat microbial mat. In general, they are K-strategists because of their slow growth. Different “lifestyle” strategies which both are typical for the microbial mat were identified within the bacterial isolates. These two at first contradictory “lifestyle” of adaptation to interface/ gradient conditions and adaptation to stable/ slowly changing environmental conditions highlight the existence of different habitats on a small area within the microbial mat.

Right now the hydrolytic enzymes seem to be of minor importance for the heterotrophic bacteria. The hydrolytic enzymes have a low affinity for their substrates and work close or even above their saturation level. Thus, the organic material turnover is very slow and organic material is accumulating within the microbial mat. Nevertheless, this does not necessary prove the unimportance of hydrolytic enzymes for the organic material turnover. Some amount of the organic material is likely excluded from the bioavailable organic material pool by attachment to sediment particles.

So far, the organic material accumulation favours the ongoing silting-up process within the wind flat The salt marsh community with *Puccinella maritima* (Hudson) Parlatore (common salt marsh grass) established itself more and more over the years. Hence, it might be a question of time until the examined microbial mat vanishes from its actual location and might colonise an until now mat-free area of the wind flat.

The special characteristics of the wind flat microbial mat due to absence of regularly mechanical disturbance by tides have to be included within future research projects. A station which surveys the water level within the wind flat was installed. First data analysis for 2010 showed the wind flat to be non-flooded most of the time, whereas flooding events with 20 – 50 cm water levels were a rare event (Karsten et al., 2010). The water level measurement is a first a first step towards a better understanding of the interaction between mechanical forces and the wind flat microbial mat as rather stable ecosystem. It could be extended by measuring flow velocity. Even when flooding of the wind flat is rare, one flooding event due to storm events might be enough to erode large parts of the microbial mat.

Furthermore, the consumer, namely protozoa like nematodes etc., have to be regarded more intensively when describing microbial mats as ecosystem. Kern (2008) did a first inventory of protozoa within the wind flat microbial mat showing the protozoa to function as additional remineralisers. So far, the importance of microfauna within microbial mats was rejected and hence is poorly analysed. The observed increasing abundance of nematodes within the wind flat microbial mat during this study now demonstrates the importance to analyse the function of protozoa for the microbial mat as ecosystem. The increasing abundance of nematodes was noticed together with the ongoing accumulation of organic material, silting-up process of the wind flat and degradation of the microbial mat. The question arises if nematodes get more abundant and hence have more influence when a microbial mat is degrading.

The degrading efficiency of hydrolytic enzymes for organic material is a general topic which has to be addressed. This study discussed the importance of hydrolytic enzymes for organic material turnover within microbial mats. Quite obvious, both hydrolytic enzyme activities and the amount and composition of the organic material are needed to get reliable results. Thus, it is important to know the hydrolytic enzymes kinetics for the analysed system. The substrate concentrations which Hoppe (1983) applied would not have been suitable for the microbial mat samples. The hydrolytic enzyme activity is so far always addressed as apparent enzyme activities. It is not known so far how many different enzymes are

expressed by the community and if these different enzymes have more or less the same characteristics (Vmax, Km etc.) or if different hydrolytic enzymes with different substrate affinity, activity etc. are expressed. An extraction and characterisation of hydrolytic enzymes from environmental samples may close the gap between classic enzymology and ecological field studies on hydrolytic enzymes. Native gel electrophoresis separates proteins by charge to mass ratio without denaturation (e.g. Kastenholz, 2004). It is possible to detect hydrolytic activity within the native gel, like it was done for glycolytic enzymes using NAD(P)H (Rivoal et al., 2002) or for microbial chitinases (Trudel and Aselin, 1989). Two microbial mat heterotrophic bacteria (*Shewanella* sp and *Pseudoalteromonas* sp) are available which represent different "lifestyle" strategies and both express α- and β-glycolytic activity. Thus it would be interesting to know, if they express different types of α- and β-glucosidase and if they express different types of α- and β-glucosidase under different substrate conditions.

The organic material itself has to be analysed in more detail as well. The microbial mat EPS is of special interest. Klock et al. (2007) developed an optimised characterisation and isolation method which allows analysis of bulk protein, neutral sugars and uronic acids. The EPS composition can be addressed in more detail to answer the question in which way the heterotrophic bacteria may benefit from the EPS mainly released by the primary producers. The HPLC-analysis applied in this study gave a first impression of the protein pool composition. Other analytic method which analyse the size, molecular weight and structure of the peptides are necessary to know how and if hydrolytic enzymes can degrade the different peptides. Analytical methods like a normal gel electrophoresis which separates peptides according to their mass can be a first step towards a better understanding of the organic material composition.

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Appendix

A. Media for cultivation of bacteria

Artificial seawater (10 PSU)

Na Cl	10 g NaCl l^{-1}

Seawater Yeast Peptone Medium

Peptone	5g l^{-1}
Yeast extract	3g l^{-1}
Artificial seawater (10 PSU)	1 l

pH: 7.8

Scource: Media 243 "Seawater Yeast Peptone Agar" (modified) of the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures

Minimal medium

Solution 1

$MgSO_4$ x 12 H_2O	0.5 g l^{-1}

Solution 2

Na-K-phosphatebuffer

KH_2PO_4	9.073 g l^{-1}	A
Na_2HPO_4 x 2 H_2O	11.87 g l^{-1}	B

53.4 portions A + 46.6 portions B

NH_4Cl	1 g l^{-1}

Solution 1 and 2 prepared with artificial seawater, autoclaved and mixed under steril conditions

Added steril filtered:

vitamins	10 ml l^{-1}
trace elements	10 ml l^{-1}
20 x $CaCl_2$	0.5 ml l^{-1}
10 x $FeNH_4$-Citrat	1 ml l^{-1}
C-scource	0.5 % (mass)

pH: 8.0

Scource: Yalleroni and Doudoroff (1972), modified

B. Ecoplate Results

ECOplate profiles for microbial mat community in May, August and October 2009. **X** shows the oxidised C-souces within 48 h **O** shows the oxidised C-scources within 9 days (n = 3).

	May 09	Aug 09	Oct 09
Carbohydrates			
Cellobiose	X		O
i-Erythritol	O	O	O
α-D-Glucose-1-Phosphate			O
D,L-α-Glycerol phosphate			
α-D-Lactose	X	O	X
D-Mannitol	X		X
β-Methyl-D-Glucoside	X		X
N-Acetyl-D-Glucosamine	X	O	X
Pyruvic acid methyl ester	X		O
Xylose		O	O
Amino acids and peptides			
L-Arginine	O	X	O
L-Asparagine	X	X	X
L-Phenylalanine	O	O	
D-Serine	O		X
L-Threonine			X
Glycyl-glutamic acid	X	O	X
Carboxylic acids			
D-Glucosaminic Acid	O		
D-Galactonic acids γ Lactone	O		O
D-Galacturonic Acid	X	O	O
2-Hydroxybenzoic Acid			
4-Hydroxybenzoic Acid			
γ-Hydroxybutyric Acid	X	O	O
Itaconic Acid		O	
α-ketobutyric acid			
D-Malic Acid	O	O	X
Polymers			
α-Cylodextrin	X		O
Glycogen	X		X
Tween 40	X	X	X
Tween 80	X	X	X
Amines/ Amides			

Phenylethylamine			X
Putrescine	X	O	X
SUM	21	15	24

C. Biolog results

Biolog profiles (GN2) for bacterial stems 9.06 B1 (*Pseudoalteromonas sp.*), 9.06 B2 (*Vibrio sp.*), 9.06 B4 (*Pseudoalteromonas sp.*), 3.07 B1 (*Bacillus baekryungensis*), 3.07 B2 (*Pseudomonas sp.*), 3.07 B3 (*Shewanella baltica*) and 3.07 B4 (*Shewanella sp.*) from wind flat microbial mat. X shows the respired C-souces (n = 1). Incubation time = 1 h.

	9.06 B1	9.06 B2	9.06 B4	3.07 B1	3.07 B2	3.07 B3	3.07 B4
Carbohydrates							
N-Acetyl-D-galactosamin	X						X
N-Acetyl-D-glucosamine	X	X	X	X	X		X
Adonitol							
L-Arabinose	X					X	
D-Arabitol							
Celliobiose	X	X		X	X		X
i-Erythritol							
D-Fructose	X	X					
L-Fucose						X	
D-Galactose		X		X		X	
Gentiobiose				X			X
α-D-Glucose	X	X	X	X	X	X	X
m-Inositol							
α-D-Lactose		X			X		
Lactulose							
Maltose	X		X		X		X
D-Mannitol	X	X				X	
D-Mannose		X			X	X	
D-Melibiose		X				X	

β-Methyl-D-Glucoside		X					
D-Psicose		X					
D-Raffinose							
L-Rhamnose							
D-Sorbitol		X	X	X	X		
Sucrose	X	X	X	X	X	X	X
D-Trehalose	X	X	X	X	X	X	
Turanose							
Xylitol							
amino acids and peptides							
D-Alanine							
L-Alanine							
L-Alanyl-glycine	X					X	X
L-Asparagine		X		X		X	X
L-Aspartic Acid						X	X
L-Glutamic acid		X				X	
Glycyl-L-Aspartic acid	X	X					X
Glycyl-Glutamic acid		X					X
L-Histidine		X				X	
Hydroxy-L-Proline						X	
L-Leucine							X
L-Ornithine							
L-Phenylalanine							
L-Proline		X					X
L-Pyroglutamic Acid						X	
D-Serine						X	X
L-Serine	X	X				X	X
L-Threonine		X					
D,L-Carnitine							
γ-Aminobutyric Acid				X		X	

Carboxylic acids							
Acetic Acid	X	X				X	X
Aconitic Acid (cis-)		X				X	X
Citric Acid	X	X				X	X
Formic Acid						X	X
D-Galactonic acid lactone							
D-Galacturonic Acid	X			X			
D-Gluconic Acid		X	X	X		X	X
D-Glucosaminic acid							
D-Glucuronic Acid						X	
α-Hydroxybutyric acid							X
β-Hydroxybutyric acid						X	X
γ-Hydroxybutyric acid							
p-Hydroxyphenylacetic acid							
Itaconic acid							
α-Ketobutyric acid							X
α-Ketoglutaric acid						X	X
α-Ketovaleric acid							
D,L-Lactic Acid		X				X	X
Malonic Acid							
Propionic Acid	X					X	
Quinic acid						X	
D-Saccharic acid						X	
Succinic Acid	X	X			X	X	X
Polymers							
Glycogen	X		X	X	X	X	X
α-Cyclodextrin	X		X	X			X
Dextrine	X	X	X	X	X	X	X
Tween 40	X	X	X	X	X	X	X
Tween 80	X	X	X	X	X	X	X

Alcohols							
2,3-Butanediol							X
Glycerol		X				X	X
Amide							
Succinamic acid							
Glucuronamide		X					
Alanineamide							
Amine							
Phenylethylalanine							
2-Aminoethanol							X
Putrscine							
Ester							
Mono-methylsuccinate						X	
Methylpyruvate							
Aromatic chemicals							
Inosine		X	X	X	X		X
Urocanic acid				X		X	
Thymidine		X					X
Uridine		X					X
Phosphorylated Chemicals							
α-D,L-Glycerol Phosphate		X					X
α-D-Glucose -1-Phosphate		X		X	X		X
D-Glucose-6-Phosphate		X					X
Brominated chemicals							
Bromosuccinic acid		X				X	X
sum	**23**	**41**	**13**	**18**	**17**	**40**	**43**

D. Exemplary calculation of organic matter turnover by hydrolytic enzyme

1. Organic matter bound in biomass

Given are

Dry mass	5.36 g
Org. content	1.13 (% DM)
Density	1.84 g cm^{-3}
Chl a	29.3 µg cm^{-3}
Bacteria	744 · 10^6 cells cm^{-3}
Molecular weight (MW) C	12.01 g mol^{-1}

1.1. Conversion of organic content (%) into organic C (mmol C cm^{-3})

A. $org.mass(g) = \frac{dryweight(g)}{100} \times org.content(DM)$

$$\frac{5.36g}{100} \times 1.13 = 0.06g(org.mass)$$

B. $50\% org.mass = org.C$

$$0.06g(org.mass) = 0.03g(org.C)$$

C. $org.C(gcm^{-3}) = \frac{org.C(g)}{demsitygcm^{-3}}$

$$\frac{0.03gC}{1.184gcm^{-3}} = 0.016gCcm^{-3}$$

D. $org.C(molcm^{-3}) = \frac{org.C(gcm^{-3})}{MWC(gmol^{-1})}$

$$\frac{0.016gCcm^{-3}}{12.01gmol^{-1}} = 1.33mmolCcm^{-3}$$

1.2. Conversion of Chl a (µg Chl a cm^{-3}) into total organic C in primary producers (Prim.Prods.) (µmol C cm^{-3})

A. 80 µg C per µg Chl a (Garnier and Maurelatos, 1991)

$$29.3\mu gChlacm^{-3} * 80\mu gC\mu gChla^{-1} = 2.344mgCcm^{-3}$$

B. $org.C(molcm^{-3}) = \frac{org.C(gcm^{-3})}{MWC(gmol^{-1})}$

$$\frac{2.344mgCcm^{-3}}{12.01gmol^{-1}} = 0.195mmolCcm^{-3}$$

1.3. Conversion of bacterial abundance (cells · 10^6 cm^{-3}) into total bacterial organic carbon (µmol C cm^{-3})

A. 10.4 fg C per bacterial cell (Simon and Azam, 1989)

$$7.44 \cdot 10^6 cells cm^{-3} * 10.4 fgC cell^{-1} = 0.744 mgC cm^{-3}$$

B. $$org.C(molcm^{-3}) = \frac{org.C(gcm^{-3})}{MWC(gmol^{-1})}$$

$$\frac{0.744 mgC cm^{-3}}{12.01 gmol^{-1}} = 0.062 mmolC cm^{-3}$$

1.4. Organic carbon bound in biomass and organic carbon degradable

A. $org.C \text{in} biomass = bacterial org.C + prim.prods.org.C$

$$0.062 mmolorg.cm^{-3} + 0.0195 mmolC cm^{-3} = 0.257 mmolC cm^{-3} \text{in} biomass$$

B. $degradeable org.C = total org.C - org.C \text{in} biomass$

$$1.33 mmolC cm^{-3} - 0.257 mmolC cm^{-3} = 1.073 mmolorg.C cm^{-3}$$

2. Turnover of proteins in microbial mat (examplary for proteins)

Given are	
Degradeable org. C	1.073 mmol cm^{-3}
Vmax Ala-aminopeptidase	1.9 µmol C cm^{-3} h^{-1}
Vmax Leu-aminopeptidase	3.5 µmol C cm-3 h-1
Vmax Phe-aminopeptidase	2.0 µmol C cm-3 h-1
Vmax Arg-aminopeptidase	0.5 µmol C cm-3 h-1
All together	7.9 µmol C cm-3 h-1

2.1. Proteins in microbial mat

$$protein(mmolC cm^{-3}) = \frac{degradeable org.C(mmolC cm^{-3})}{100\%} * protein(\%)$$

35 – 58 % of POC are proteins (Schumann et al., 2001)

$$\frac{1073 mmolC cm^{-3}}{100\%} * 35\% = 0.376 mmolC Protein cm^{-3}$$

2.2. Turnover of proteins

$$turnover(h) = \frac{protein(mmolC\,cm^{-3})}{Vmaxaminopeptidase(mmolC\,cm^{-3}h^{-1})}$$

$$\frac{0.376 mmolC\,cm^{-3}}{0.0079 mmolC\,cm^{-3}h^{-1}} = 48h$$

3. Amino acids in microbial mats (HPLC)

Given are	
Peak area of Ala-standard (50 nmol l^{-1})	421.4
C-atoms of Alanine	3
Peak area of internal standard (IS) α-ABA (50 nmol l^{1})	1826
Peak area of Ala in sample(DFAA)	1897
Peak area of IS in sample	39.1
Dry mass	0.505 g
Density	2.2 g cm^{-3}
Additional volume for extraction*	2 ml

* s. Material and methods for details

3.1. Evaluation of HPLC-results into concentration of amino acids in sample (nmol l^{-1})

A. $$responsefactor(RF) = \frac{peakareaofAA\text{in}standard}{peakareaofinternal(IS)standard\text{in}standard}$$

$$\frac{241.4}{1826} = 0.231 RFofAla$$

B. $$AA(nmoll^{-1}) = \frac{peakareaAA\text{in}sample*concentrationIS}{peakareaIS\text{in}sample*RF}$$

$$\frac{1897 * 50 nmoll^{-1}}{31.1 * 0.231} = 10501 nmolAlal^{-1}$$

3.2. Amino acids in microbial mat (nmol C cm-3)

A. $$sedimentvolume(cm^3) = \frac{drymass(g)}{density(gcm^{-3})}$$

$$\frac{0.505g}{2.2gcm^{-3}} = 0.23cm^3$$

B. $$sampledilution* = \frac{sedimentvolume(cm^3)+additionalvolume(ml)}{sedimentvolume(cm^3)}$$

* due to extraction (s. Material and Methods for details)

$$\frac{0.23cm^3 + 2ml}{0.23cm^3} = 9.7$$

C. $totalvolume(l) = \frac{sedimentvolume(cm^3)+additionalvolume(ml)}{1000}$

$$\frac{0.23cm^3 + 2ml}{1000} = 0.00223l$$

D. $AA(nmolCcm^{-3}) = \frac{AA(nmoll^{-1})*sampledilution*totalvolume(l)}{sedimentvolume(cm^3)} * CatomsAA$

$$\frac{10501nmolAlal^{-1} * 9.7 * 0.00223l}{0.23cm^3} * 3 = 2964nmolCcm^{-3}$$

4. Turnover of amino acids in microbial mat

Given are

Chl a:	0.00182 mmol C cm^{-3}
Bacteria:	0.064 mmol C cm^{-3}
Protein/ Chl a	14 (Valenzueale-Espinoza et al., 2001)
THAA:	0.42 mmol C cm^{-3}
DFAA:	0.076 mmol C cm^{-3}
Vmax Ala-aminopeptidase	1.9 µmol C cm^{-3} h^{-1}
Vmax Leu-aminopeptidase	3.5 µmol C cm^{-3} h^{-1}
Vmax Phe-aminopeptidase	2.0 µmol C cm^{-3} h^{-1}
Vmax Arg-aminopeptidase	0.5 µmol C cm^{-3} h^{-1}
All together	7.9 µmol C cm^{-3} h^{-1}

4.1. Protein in primary producers

$$0.00182mmolCcm^{-3} * 14 = 0.025mmolCcm^{-3}$$

4.2. Protein in bacteria

60 % of bacterial C is protein (Simon and Azam, 1989)

$$bacterialProtein(mmolCcm^{-3}) = \frac{bacterialC(mmolCcm^{-3})}{100\%} * 60\%$$

$$\frac{0.064mmolCcm^{-3}}{100\%} * 60\% = 0.039mmolCcm^{-3}$$

4.3. Degradable protein in microbial mat

$$degradableprotein(mmolCcm^{-3}) = THAA(mmolCcm^{-3}) - DFAA(mmolCcm^{-3}) - proteinin Prim.Prods.(mmolCcm^{-3}) - bacterialprotein(mmolCcm^{-3})$$

$$0.422mmolCcm^{-3} - 0.076mmolCcm^{-3} - 0.039mmolCcm^{-3} - 0.025mmolCcm^{-3} = 0.29mmolCcm^{-3}$$

4.4. Turnover of degradable protein in microbial mat

$$turnover(h) = \frac{degradableprotein(mmolCcm^{-3})}{Vmaxaminopeptidases(mmolCcm^{-3}h^{-1})}$$

$$\frac{0.29mmolCcm^{-3}}{0.0078mmolCcm^{-3}h^{-1}} = 36h$$

E. Methodolgical details for sequencing of bacteria isolates from wind flat microbial mat

- cells were harvested after 48 h cultivation by centrifugation (13.000rpm; Heraeus Biofuge pico)
- washed twice with phosphate buffered saline (PBS)
- DNA was extracted by freeze and thaw-method: Cells were frozen at -20 °C for 2 h and then boiled for 4 minutes and resuspended in 50 µl diethylpyrocarbonate (DEPC) water.
- 16SrDNA was amplified by Polymerase Chain Reaction (PCR) with primers Bac27f (5' AGAGTTTGATCMTGGCTCAG) and Bac1492r (5' GGYTACCTTGTTACGACTT)
- PCR products were separated by gel electrophoresis (1 % agarose with ethidiumbromide; 3 µl Ladder (λDNA/ EcoRI+Hind III); 3 µl Loading Dye + 25 µl PCR-products)
- Bands were purified by GFX™ PCR DNA and Gel Band Purification Kit
- sequenced by Qiagen and compared with databases for identification.

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G. Selbstständigkeitserklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe. Dazu habe ich keine außer den von mir angegebenen Hilfsmitteln und Quellen verwendet und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen habe ich als solche kenntlich gemacht.

_____

Kerstin Heyl

Rostock, 24. November 2015