DISS. ETH NO. 20685

BACTERIAL CHITIN AND AMINO-COMPOUND DEGRADATION IN TWO CONTRASTING LAKES

A dissertation submitted to

ETH ZURICH

for the degree of

Doctor of Sciences

presented by

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2012
TABLE OF CONTENTS

Summary ......................................................................................................................1

Zusammenfassung .......................................................................................................3

1. Introduction ...................................................................................................5
   1.1. Motivation ...................................................................................................5
   1.2. Chitin and chitin degradation .................................................................7
   1.3. Peptidoglycan ..........................................................................................9
   1.4. Degradation indices ............................................................................11
   1.5. Lake ecosystem ....................................................................................12
   1.6. Study sites ............................................................................................14
   1.7. Molecular methods ............................................................................15
   1.8. Objectives and outline of the thesis ..................................................17

2. Bacterial chitin hydrolysis in two lakes of contrasting trophic statuses21
   2.1. Abstract ...................................................................................................22
   2.2. Introduction ...........................................................................................22
   2.3. Methods ..................................................................................................24
       2.3.1. Sampling sites ................................................................................24
       2.3.2. Sampling water and sediments ....................................................25
       2.3.3. Zoo- and phytoplankton communities .....................................25
       2.3.4. Zooplankton chitin ..................................................................26
       2.3.5. Chemical analysis ......................................................................26
       2.3.6. Chitinase activity ........................................................................27
       2.3.7. DNA extraction ..........................................................................29
       2.3.8. Amplification and quantification of chitinase gene fragments ......30
   2.4. Results .....................................................................................................31
       2.4.1. Biogeochemistry of lake water columns ..................................31
       2.4.2. Biogeochemistry of sediment profiles ......................................33
       2.4.3. Zoo- and phytoplankton community composition .....................34
       2.4.4. Zoo and- phytoplankton biomass .............................................34
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.5. Zooplankton chitin</td>
<td>35</td>
</tr>
<tr>
<td>2.4.6. Zooplankton chitin biomass compared to TOC and GlcN concentrations</td>
<td>35</td>
</tr>
<tr>
<td>2.4.7. Zooplankton chemistry</td>
<td>36</td>
</tr>
<tr>
<td>2.4.8. Chitinase activity</td>
<td>36</td>
</tr>
<tr>
<td>2.4.9. Chitinase gene copies</td>
<td>38</td>
</tr>
<tr>
<td>2.4.10. Correlations between chitinase activity, chiA abundance, and biogeochemical parameters</td>
<td>41</td>
</tr>
<tr>
<td>2.5. Discussion</td>
<td>43</td>
</tr>
<tr>
<td>2.5.1. Sources of chitin and glucosamine in freshwater lakes</td>
<td>43</td>
</tr>
<tr>
<td>2.5.2. Chitinolytic activity and populations in freshwater lakes</td>
<td>44</td>
</tr>
<tr>
<td>3. Rare bacterial community members dominate the functional trait of chitin hydrolysis in freshwater lakes</td>
<td>47</td>
</tr>
<tr>
<td>3.1. Abstract</td>
<td>48</td>
</tr>
<tr>
<td>3.2. Introduction</td>
<td>48</td>
</tr>
<tr>
<td>3.3. Methods</td>
<td>51</td>
</tr>
<tr>
<td>3.3.1. Sampling</td>
<td>51</td>
</tr>
<tr>
<td>3.3.2. DNA extraction</td>
<td>52</td>
</tr>
<tr>
<td>3.3.3. 454 pyrosequencing</td>
<td>52</td>
</tr>
<tr>
<td>3.3.4. Data pre-processing and analysis of 16S rRNA gene sequences</td>
<td>54</td>
</tr>
<tr>
<td>3.3.5. Data pre-processing and analysis of chiA sequences</td>
<td>55</td>
</tr>
<tr>
<td>3.4. Results</td>
<td>56</td>
</tr>
<tr>
<td>3.4.1. Bacterial community structure</td>
<td>56</td>
</tr>
<tr>
<td>3.4.2. OTU-based diversity analyses of 16S rRNA gene and chitinase sequences</td>
<td>58</td>
</tr>
<tr>
<td>3.4.3. Similarity of 16S rRNA gene and chitinase OTUs between diverse lake habitats</td>
<td>62</td>
</tr>
<tr>
<td>3.4.4. Chitinases detected in diverse lake habitats</td>
<td>63</td>
</tr>
<tr>
<td>3.5. Discussion</td>
<td>67</td>
</tr>
</tbody>
</table>
4. Impact of amino compounds and organic matter degradation state on the vertical structure of lacustrine bacteria ...............................................................73

4.1. Abstract.................................................................74
4.2. Introduction.................................................................74
4.3. Methods .................................................................77
  4.3.1. Sampling sites ................................................77
  4.3.2. Sampling ........................................................77
  4.3.3. Chemical analysis .............................................77
  4.3.4. Amino acid analysis .........................................78
  4.3.5. Degradation index .............................................78
  4.3.6. Amino sugar analysis ........................................78
  4.3.7. Chlorin Index ..................................................78
  4.3.8. Bacterial cell counts .........................................79
  4.3.9. DNA extraction ...............................................79
  4.3.10. Amplification of ribosomal intergenic spacer fragments .........79
  4.3.11. Binning ........................................................80
  4.3.12. Statistical analysis ...........................................80
4.4. Results .................................................................................83
  4.4.1. Richness and community structure of free-living and particle-associated bacteria..................................................83
  4.4.2. Amino acids ......................................................85
  4.4.3. Amino sugars ....................................................86
  4.4.4. Chlorin Index ....................................................87
  4.4.5. Explanatory variables with the strongest influence on between- and within-lake variability of the bacterial community structure ..........88
  4.4.6. Bacterial cell abundance .......................................92
  4.4.7. Multiple regression analysis ................................93
4.5. Discussion..............................................................................96

5. Contribution of bacterial cells to lacustrine organic matter based on amino sugars and D-amino acids.................................................................101

5.1. Abstract..............................................................................102
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.</td>
<td>Introduction</td>
<td>102</td>
</tr>
<tr>
<td>5.3.</td>
<td>Methods</td>
<td>104</td>
</tr>
<tr>
<td>5.3.1.</td>
<td>Sampling sites</td>
<td>104</td>
</tr>
<tr>
<td>5.3.2.</td>
<td>Sampling</td>
<td>104</td>
</tr>
<tr>
<td>5.3.3.</td>
<td>Chemical analysis of water samples</td>
<td>105</td>
</tr>
<tr>
<td>5.3.4.</td>
<td>Chemical analysis of particulate organic matter</td>
<td>106</td>
</tr>
<tr>
<td>5.3.5.</td>
<td>Bacterial cell counts</td>
<td>108</td>
</tr>
<tr>
<td>5.4.</td>
<td>Results</td>
<td>108</td>
</tr>
<tr>
<td>5.4.1.</td>
<td>Physico-chemical characterization of the water columns</td>
<td>108</td>
</tr>
<tr>
<td>5.4.2.</td>
<td>Total amino sugar concentrations</td>
<td>110</td>
</tr>
<tr>
<td>5.4.3.</td>
<td>Amino sugars in POM</td>
<td>112</td>
</tr>
<tr>
<td>5.4.4.</td>
<td>D-Amino acids in POM</td>
<td>115</td>
</tr>
<tr>
<td>5.4.5.</td>
<td>Bacterial cell counts</td>
<td>116</td>
</tr>
<tr>
<td>5.5.</td>
<td>Discussion</td>
<td>117</td>
</tr>
<tr>
<td>5.5.1.</td>
<td>Origin and transformation processes of amino sugars in the water column</td>
<td>117</td>
</tr>
<tr>
<td>5.5.2.</td>
<td>Contribution of bacterial cells to the organic carbon pool in the water column</td>
<td>119</td>
</tr>
<tr>
<td>5.5.3.</td>
<td>Effects of trophic status and redox conditions on amino sugar transformation and bacterial contributions to the organic carbon pool</td>
<td>122</td>
</tr>
<tr>
<td>6.</td>
<td>Conclusions and outlook</td>
<td>125</td>
</tr>
<tr>
<td>6.1.</td>
<td>Sites and significance of chitin hydrolysis</td>
<td>125</td>
</tr>
<tr>
<td>6.2.</td>
<td>Bacterial chitinases</td>
<td>126</td>
</tr>
<tr>
<td>6.3.</td>
<td>Organic matter degradation indices and bacterial dynamics</td>
<td>128</td>
</tr>
<tr>
<td>6.4.</td>
<td>Bacterial contribution to organic matter</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>Acknowledgments</td>
<td>153</td>
</tr>
</tbody>
</table>
SUMMARY

One crucial function of aquatic bacteria is the degradation of recalcitrant organic matter, which is not readily assimilable by aquatic organisms of higher trophic levels. Via its incorporation as bacterial biomass, carbon and nutrients are reintroduced into the aquatic food web. For instance, the function of breaking down the biopolymer chitin in aquatic ecosystems is mainly assigned to bacteria. Chitin is a homopolymer of the amino sugar N-acetylg glucosamine and is synthesized as structural component by fungi, arthropods, and algae. The total aquatic annual chitin production is estimated at $10^{10}$ to $10^{12}$ kg chitin year$^{-1}$. If this huge amounts of insoluble carbon and nitrogen were not converted to biologically useful material, the hydrosphere would get depleted of these elements. Compared to the number of studies in marine systems, studies on the degradation of freshwater chitin are rare. This thesis aimed to study the role of lacustrine bacteria in the degradation of particulate amino compounds focusing on the hydrolysis of chitin. Two temperate lakes distinguishing themselves by their trophic and redox statuses were selected as study sites: Lake Brienz is oligotrophic and fully oxic while Lake Zug is eutrophic and partially anoxic.

In chapter 2, the the main sites of chitin hydrolysis were studied by measuring the turnover rates of the chitin analog methylumbelliferyl-$N,N'$-diacetetylchitobioside (MUF-DC) and the presence of chitinase genes ($chiA$) in zooplankton, the water columns, and the upper seven centimeter of the sediments. The zooplankton and the sediments were identified as the main sites of chitin hydrolysis. Although no chitinolytic activity could be measured in the lake water columns, significant $chiA$ copy numbers were detectable, especially in the oligotrophic water column. These findings suggest a higher significance of chitin as bacterial growth substrate in the oligotrophic than in the eutrophic system and that the chitinolytic activity is mainly associated with particulate fractions providing the free-living bacterial communities with chitin hydrolysis products.

In order to identify the chitinolytic bacteria in samples for which high $chiA$ concentrations were detected, high-throughput sequencing of $chiA$ and 16S rRNA gene fragments was applied (chapter 3). The results revealed distinct diversity and distribution of bacterial chitinases between lakes and habitats, which suggests that the chitinolytic bacteria are a functional group that is strongly shaped by the environmental parameters and ecological conditions of its habitat. The detected
chitinases could be assigned to chitinases of *Stenotrophomonas maltophilia*, *Janthinobacterium lividum* and *Actinobacteria*. However, the predominant chitinases of Lake Zug represent a novel bacterial chitinase lineage. Concluding from the abundance of the potentially chitinolytic bacteria in the total bacterial communities, i.e., the number 16S rDNA sequences detected, the chitinolytic bacteria appeared as rare members of the bacterial communities in the diverse lake habitats. Their presence is proposed to be essential for the functional trait of chitin degradation in freshwater lakes.

In chapter 4, the influence of degradation state and composition of particulate organic matter on the structural shifts of the total bacterial communities was studied. For this purpose, changes in the composition of the free-living and particle-associated bacteria along the lake water columns were analyzed. Whereas amino compound based degradation indices performed less well as indicators for bacterioplankton dynamics, the Chlorin Index, a measure for the degradation state of particulate organic matter from primary production, was identified as one of the main predictors of the vertical shifts in the abundance and composition of bacterioplankton.

In chapter 5, the proportion of organic matter originating from bacteria themselves was estimated using cell counts and the bacterial amino biomarkers muramic acid and D-amino acids. The results consistently showed a higher proportion of bacterial derived organic carbon in oligotrophic Lake Brienz compared to eutrophic Lake Zug. Furthermore, higher turnover rates of particulate amino sugars were detected in Lake Brienz.

In conclusion, this thesis revealed a higher significance of bacteria for the degradation and transformation of particulate amino compounds in an oligotrophic than in a eutrophic lake. In particular, the amino sugar polymer chitin was found as a relatively more important growth substrate in the oligotrophic system, where other readily available organic substrates are scarce. Its hydrolysis appeared restricted to a limited number of bacterial chitinases, which are assumed to be crucial for the recycling of carbon and nitrogen bound as chitin. Future studies surveying a wide range of lakes could show if similar chitinolytic bacteria are distributed between systems of similar trophic state and if the functional trait of chitin hydrolysis is generally more significant in oligotrophic compared to eutrophic systems.


Die chitinabbauenden Bakterien wurden mittels Sequenzierung der bakterielle 16S rDNA und des $chiA$ Gens in Proben mit hoher $chiA$ Konzentration identifiziert (Kapitel 3). Die detektierten bakteriellen Chitinase zeigten unterschiedliche Diversität und Verbreitung zwischen den Seen und den See-Habitaten, was auf eine


1. INTRODUCTION

1.1. Motivation

A crucial function of aquatic bacteria and microorganisms in general is the decomposition of organic matter and the resulting recycling of nutrients and carbon (Azam et al. 1983, Sherr and Sherr 1991). Approximately 30-60% of the planktonic primary production is mineralized by heterotrophic bacteria (Biddanda et al. 1994, Del Giorgio et al. 1997). In aquatic ecosystems, primary producers like algae and cyanobacteria produce organic compounds by photosynthetic carbon fixation (Fig. 1). Eukaryotic microorganisms such as flagellates graze on small-sized primary producers and on heterotrophic bacteria. The flagellates are in turn preyed upon by microzooplankton such as ciliates. Larger zooplankton feeds on the microzooplankton and phytoplankton, which transfers the organic matter from primary production up the food web. After an organism’s death and processes like cell senescence, but also through bacterial lysis (e.g., after viral infection), exudation of exopolymers from phytoplankton, and excretion of waste products (e.g., fecal pellets, exuviae), large amounts of particulate and dissolved organic matter are released into the aquatic environment. A high fraction (~50%) of the aquatic organic material consists of polymeric, high molecular weight compounds, which are not readily assimilable by aquatic organisms of higher trophic levels (Allen 1976, Benner et al. 1992, Tulonen et al. 1992). Heterotrophic bacteria are able to decompose this recalcitrant organic matter and reintroduce it into the aquatic food web via its incorporation as bacterial biomass (microbial loop) (Middelboe et al. 1995). In particular, bacteria are able to degrade the polymeric, high molecular weight compounds to their monomers, which can easily be transported across cell membranes. For instance, the function of breaking down the biopolymer chitin, which is composed of the amino sugar glucosamine (Fig. 2), is mainly assigned to bacteria (Zobell and Rittenberg 1937, Cottrell and Kirchman 2000, Beier and Bertilsson 2011). Chitin is synthesized as structural component by a wide range of aquatic organisms and, therefore, highly abundant in aquatic ecosystems (Cauchie 2002).
Bacteria build up new biomass and are, therefore, themselves sources of complex organic matter. For instance, the structural component of the bacterial cell wall consists of peptidoglycan, which is unique to bacteria. Peptidoglycan is made up of a polysaccharide of amino sugars (ASs) cross-linked by short peptides consisting of alternating D- and L-amino acids (AAs) (Fig. 3). Another large component of polymeric, high molecular weight organic matter is represented by proteins. Proteins are the major nitrogenous compounds in all living organisms and built up from L-AAs.

![Eutrophic zone: Primary production](image)

**Fig. 1.** Fate of organic nitrogen compounds in an aquatic ecosystem focusing on the bacterial degradation of chitin. Bacteria not only degrade organic matter, they build up new biomass and are, therefore, themselves, source of organic matter. AAs, amino acids; ASs, amino sugars; GlcNAc, N-acetylglucosamine; GlcN, glucosamine.

The production, degradation and preservation of amino compounds by aquatic biota may impact both global carbon and nitrogen cycles. Organic matter which is not
decomposed in the water column is deposited in the sediments and mineralized by the sedimentary microorganisms or buried. Studies on the role of lakes and reservoirs as carbon sinks have shown that inland waters store organic carbon more efficiently compared to marine systems (Mulholland and Elwood 1982, Dean and Gorham 1998). The high carbon burial efficiency of lake sediments compared to marine sediments was attributed to the higher input of allochthonous organic matter to lakes and was shown to be strongly negatively related to the oxygen exposure time (Sobek et al. 2009). As anoxic bottom-water is more prevalent in lakes than in oceans, the organic carbon preservation is favored in lake sediments (Battin et al. 2009). The high organic carbon burial efficiency, together with reviews of data for CO₂ and CH₄ emission from lakes and reservoirs have led to a revised view of inland waters as active components of the global carbon cycle rather than passive pipes that transport carbon to the oceans (Cole et al. 2007, Battin et al. 2009, Tranvik et al. 2009, Bastviken et al. 2011).

The aim of this thesis was to study the role of bacteria in the degradation of particulate amino compounds in a fully oxic, oligotrophic lake compared to a partially anoxic, eutrophic lake. The focus was mainly set on the bacterial hydrolysis of chitin. To estimate the proportion of bacterial derived organic matter in the lakes under study, bacterial amino biomarkers abundant in the unique bacterial cell wall polymer peptidoglycan were used. In the following chapters, chitin, the unique bacterial cell wall polymer peptidoglycan, and various parameters indicating degradation of organic matter are introduced. The lake ecosystem in general, the study sites and the methods which were applied to analyze the lacustrine bacterial communities are also described.

1.2. Chitin and chitin degradation

Chitin is a homopolymer of β-1,4-linked N-acetylatedglucosamine (GlcNAc) residues (Fig. 2), which can be arranged in antiparallel (α), parallel (β), or mixed (γ) strands. The antiparallel α configuration of the GlcNAc strands is the most tightly packed and most commonly found structure of chitin in organisms, probably as it is more difficult to break down (Gooday 1990). Except for the chitin of diatoms, known as chitan, chitin is always found crosslinked to other structural components (glucans, proteins) (Smucker 1991). The degree of deacetylation can also vary from 0 to 100% (chitosan) (Gooday 1990).
Chitin synthesis is widely distributed among phyla. It is a structural component of the cell wall of fungi and the exoskeleton of invertebrates but is also found in protozoa (Mulisch 1993) and algae (Herth 1978, Kapaun and Reisser 1995). With the exception of Streptomyces spore walls, chitin was not found in prokaryotes (Gooday 1990). Chitin is one of the most abundant biopolymers on earth. The annual production and the steady state amount in the biosphere is on the order of $10^{12}$ to $10^{14}$ kg (Jeuniaux and Voss-Foucart 1991, Poulicek et al. 1998). On the basis of literature data on chitin production of arthropods, the total annual chitin production in aquatic environments was estimated at $2.8 \times 10^{10}$ kg chitin year$^{-1}$ for freshwater ecosystems and $1.3 \times 10^{12}$ kg chitin year$^{-1}$ for marine ecosystems (Cauchie 2002). But also algae are supposed to constitute a major source of chitin in an aquatic system during algal blooms. In the diatom *Thalassiosira fluviatilis*, e.g., chitin was found to represent 31-38% of the total cell mass (including the silica) (McLachlan et al. 1965).

Not only phyto- and zooplankton and insect carcasses, but also zooplankton molting (exuviae) and excretion of fecal pellets (peritrophic membranes) contribute to the production of huge amounts of chitinous particles in the water column (Turner 2002). These chitinous particles are part of the marine or lake snow which was shown to represent a hotspot of particulate organic matter solubilization (Simon et al. 1993, Grossart and Simon 1998a, b).

If this enormous quantity of carbon and nitrogen bound in form of insoluble chitin was not converted to assimilable material, the hydrosphere would be depleted of these elements in decades (Keyhani and Roseman 1999). Zobell and Rittenberg...
1.3 Peptidoglycan

The unique bacterial cell wall biopolymer peptidoglycan consists of a repeating disaccharide, β-1,4-bonded GlcNAc and N-acetylmuramic acid, the latter amino sugar is unique to bacteria (Schleifer and Kandler 1972). In Gram-negative bacteria peptidoglycan comprises less than 10% of the cell wall, whereas it can constitute up to 70% of the cell wall of Gram-positive bacteria (Schleifer and Kandler 1972). The polysaccharide chains are cross-linked by small peptides consisting of...
both L- and D-AAs (Fig. 3). The composition and number of AAs vary depending on the organism: Gram-negative bacteria and Gram-positive bacilli have meso-diaminopimelic acid (DAP) as the third amino acid (DAP-type peptidoglycan), whereas most other Gram-positive bacteria have L-lysine as the third amino acid (Lys-type peptidoglycan) and a glycine-interbridge between the peptide chains (Fig. 3) (Royet and Dziarski 2007). Both N-acetylmuramic acid and D-AAs have been used as biomarkers for bacterial biomass (Kaiser and Benner 2008, Kawasaki et al. 2011).

![Fig. 3. Structure of meso-diaminopimelic acid (DAP)-type and L-lysine (Lys)-type peptidoglycan (Royet and Dziarski 2007).](image)

In the ocean, bacterial cell components were found to contribute ~25% of particulate and dissolved organic carbon and ~50% of particulate and dissolved organic nitrogen (Kaiser and Benner 2008), thus, bacterial cell wall material represents a substantial fraction of the aquatic organic matter. After bacterial death, peptidoglycan can serve as bacterial growth substrate (Jørgensen et al. 2003). Based on radiolabeled cell wall components, the turnover of detrital peptidoglycan in seawater was estimated at 10-167 d, however, this represented a up to 21 times lower rate than that of the bacterial protein (Nagata et al. 2003). The remineralization rate of the peptide component of peptidoglycan was found to be three times higher than that of the polysaccharide components (Nagata et al. 2003).
1.4. Degradation indices

As some amino compounds are preferentially degraded, particular amino compounds become enriched compared to others during organic matter degradation. Therefore, the relative abundance of specific AAs and ASs and ratios thereof can be used to determine the diagenetic state of particulate organic matter (Lee and Cronin 1984, Haake et al. 1992, Haake et al. 1993, Dauwe and Middelburg 1998). For instance, in marine organic matter enriched in chitin, ratios between the ASs glucosamine (GlcN) and galactosamine (GalN) of > 8 and decreasing GlcN:GalN ratios during biodegradation were detected (Müller et al. 1986, Liebezeit 1993, Benner and Kaiser 2003). The ASs GlcN and GalN are more resistant to decomposition than AAs (Dauwe and Middelburg 1998) and particulate organic matter was found enriched in AAs relative to ASs during decomposition in marine water columns (Ittekkot et al. 1984, Müller et al. 1986, Haake et al. 1992). Among AAs, particular AAs are preferentially degraded. For instance, in marine particulate matter, the AAs glycine, serine, and threonine were found enriched in degraded material relative to apparently more labile AAs, such as the AAs tyrosine, phenylalanine, and glutamic acid (Lee and Cronin 1984, Dauwe and Middelburg 1998, Dauwe et al. 1999).

Based on observed systematic changes in the mole percent contribution of AAs with progressive degradation of marine particulate matter, the degradation index DI was developed (Dauwe and Middelburg 1998, Dauwe et al. 1999). The DI is calculated based on the first axis of a principle component analysis of mole percentages of the detected particulate protein AAs. For marine particulate matter highest DI values (+1) were found for fresh, unaltered organic matter (plankton samples) and decreasing DI values (towards negative values) for altered and refractory material (Dauwe et al. 1999). More recent studies indicate that the DI is most applicable to record later stages of organic matter decay in sediments (Meckler et al. 2004, Unger et al. 2005).

A different measure for the degradation state of organic matter from primary production is the Chlorin Index (CI) introduced by Schubert et al. (2005). It is based on fluorescence measures of chlorophyll pigments in a sample. The CI is defined as the fluorescence intensity of chemically modified chlorophyll (acidified with 25% hydrochloric acid) divided by the fluorescence intensity of the non-acidified original
chlorophyll extract. The CI of fresh chlorophyll \( a \) is 0.2 and increases up to unity for highly degraded organic matter from primary production. The CI has been proven to be a powerful tool to characterize the initial stages of organic matter decay in lakes (Meckler et al. 2004, Bechtel and Schubert 2009).

### 1.5. Lake ecosystem

Lakes are inland water bodies and are extremely diverse in size and character. The deepest lake is Lake Baikal with a maximum depth of 1’637 m. On earth, there are estimated 304 million natural lakes (including natural ponds \( \geq 0.001 \text{ km}^2 \)), the majority of which are small water bodies – only 17’357 lakes have a surface area of at least 10 km\(^2\) (Downing et al. 2006). Lakes and ponds together constitute about 2.8% of the non-oceanic land area (Downing et al. 2006). In contrast, marine systems cover about 71% of the earth’s surface and contain about 97% of the earth’s water (Hughes 1999). The oceans generate 32% of the earth’s net primary production. In comparison, freshwater systems generate about 3% of the earth’s net primary production and represent only 0.009% of the earth’s total water (Hughes 1999). The net primary production is the gross primary production (photosynthetic assimilation of atmospheric CO\(_2\)) minus respiratory losses.

A significant feature of lakes is the vertical stratification of physicochemical conditions, at least during a certain period of the year. Most temperate lakes are thermally stratified during the summer months (Fig. 4). The warmer, low density surface waters, the epilimnion, overlie the cooler, denser waters of the hypolimnion below. The epilimnion and the hypolimnion are separated by a thermocline, which constitutes a steep temperature gradient that exists in the middle zone of the lake, the metalimnion. Waters within the epilimnion are turbulently mixed by wind action. The upper water layers of the water column receive the most sunlight and, therefore, the phytoplankton is concentrated in the surface waters. The photosynthetic activities of the phytoplankton together with O\(_2\) diffusion from the atmosphere cause the surface water layers to be well oxygenated. In contrast, the hypolimnion develops stagnant conditions and becomes depleted in oxygen due to microbial decomposition of organic material sedimenting from the surface layers. In productive, e.g., eutrophic, lakes the hypolimnion may become anoxic during the later stages of stratification (summer) because of the high rate of microbial respiration (Sigg et al. 1991). Many
highly productive deep lakes are permanently stratified, known as meromictic lakes. But meromixis also occurs due to water density differences caused by a steep gradient in salt concentrations (chemocline). For instance, the monimolimnion, i.e., the dense bottom stratum that does not intermix with the water above, of the alpine Lake Cadagno constantly receives salt-rich water from subaquatic springs, whereas the water layers above are fed by electrolyte-poor surface water (Del Don et al. 2001). The stratification of temperate lakes typically breaks down in the fall as surface temperatures cool and the lake circulates from top to bottom, which may result in a re-oxygenation of the former hypolimnion and the oxidation of reduced compounds. In winter, inverse stratification may occur, with colder water or ice overlying warmer and denser waters below. The next lake turnover occurs in spring, when the lake’s surface is warmed up, breaking up temperature differences again and the lake water column mixes from top to bottom.

Fig. 4. Seasonal stratification in temperate lakes.

Compared to oceans, many lakes, especially small lakes, receive high loadings of organic matter from the surrounding landscape (allochthonous organic matter) (Wetzel 1995). Thus, system respiration exceeds gross primary production in many lakes (Cole et al. 2000) and allochthonous carbon was found to significantly contribute to lacustrine food webs. $^{13}$C dynamics of two American lakes indicated 40-55% of particulate organic carbon and 22-50% of zooplankton carbon derived from terrestrial sources (Pace et al. 2004). Allochthonous material often has a higher degree of recalcitrance compared to the endogenously produced (autochthonous) organic matter (Hanson et al. 2011). The high input of allochthonous organic matter was also found one significant reason for the higher organic carbon burial efficiency in lake sediments compared to marine sediments (Sobek et al. 2009). The high organic carbon burial efficiency observed in lakes has contributed to a revised view of inland waters as active components of the global carbon cycle (Cole et al. 2007).
The distinct characteristics of marine and freshwater systems may explain the evolution of bacterial clades restricted to either freshwater or marine habitats (Methe et al. 1998, Glöckner et al. 2000, Zwart et al. 2002). A fundamental difference in the microbiology is the prominence of β-Proteobacteria in freshwater versus their near absence from the oceans. Zwart et al. (2002) suggested salinity, which would require multiple adaptions to maintain effective osmotic regulation and protein conformation, as the main determinant for this finding (Zwart et al. 2002). This is supported by a recent study which identified salinity as the major driver not only for the distribution of bacterial communities but also of bacterial chitinase proteins (Beier et al. 2011).

1.6. Study sites

In order to determine the impact of distinct environmental parameters on the degradation of sedimenting particulate amino compounds, two deep temperate freshwater lakes of contrasting characteristics were selected as study sites (Fig. 5). Lake Brienz represents a nutrient-poor (oligotrophic) and Lake Zug a nutrient-rich (eutrophic) lake.

![Fig. 5. Map of Lake Brienz and Lake Zug and sampling sites (reproduced with permission of swisstopo /JA100119).](image)
Besides the distinct trophic statuses, Lake Brienz has a fully oxic water column, whereas the sampled Southern Basin of Lake Zug is permanently stratified (meromictic) with a deep anoxic water body. Lake Zug lacks of large in- and outflows which results in a long mean water residence time of approximately 14 years (Moor et al. 1996). In contrast, Lake Brienz is drained by the major rivers Aare and Lütschine, which enter the lake at opposite ends. However, recent studies on the lipid biomarker composition of the particulate organic matter in the water column and of the organic matter in the sediments revealed that the organic matter in Lake Brienz mostly derives from autochthonous sources like algae, zooplankton and bacteria (Bechtel and Schubert, 2009 a, b). Overall these two lakes represent end members in the trophic state of deep lakes in Switzerland. Choosing these lakes should allow to also determine the range of the processes studied in detail in this thesis.

In this thesis, microbial and biogeochemical data were linked to elucidate if the distinct trophic and redox statuses of the lakes result in distinct dynamics of bacterial organic matter transformation. The bacteria involved in the degradation and transformation of particulate amino compounds, in particular the amino sugar polymer chitin, were analyzed using molecular methods.

### 1.7. Molecular methods

Comparing the number of microbial cells cultivated on growth medium plates with total direct microscopic counts revealed high discrepancies, known as the “great plate count anomaly”, and indicated that only less than 1% of the bacteria in nature can be cultivated (Staley and Konopka 1985, Amann et al. 1995). The advent of molecular biology which targets the ubiquitous cell macromolecules, RNA or DNA, was a great step to gain a more complete picture of the microbial diversity in an environment. Molecular microbial biologists frequently use the ribosomal 16S rRNA molecule as a general microbial marker, as it is present in high copy numbers in all prokaryotes, contains conserved and variable regions and large 16S rRNA sequence databases are available for comparison and phylogenetic analysis (Hugenholtz 2002). Analysis of functional genes has elucidated the ecological role of the observed organisms in an ecosystem (Costello and Lidstrom 1999, Minz et al. 1999, Cébron et al. 2004).
In this thesis, the molecular fingerprinting technique Automated Ribosomal Intergenic Spacer Analysis (ARISA) was applied to follow the dynamics of the bacterial community structure along the lake water columns. The internal transcribed spacer (ITS) regions between the 16S and 23S ribosomal genes have a higher heterogeneity in both length and nucleotide sequence compared to the flanking genes and are, therefore, suitable for high resolution profiling of microbial communities (Nocker et al. 2007). ARISA detects the species-specific length variation of amplified ITS fragments by using an automated sequencing system.

In order to quantify the genetic potential of the bacterial communities to degrade chitin, quantitative real-time PCR (qPCR) was applied. In contrast to conventional PCR, real-time PCR allows for the detection of the accumulation of amplified DNA product as the PCR reaction progresses, in “real time” (Higuchi et al. 1992, Higuchi et al. 1993). For this purpose, DNA-binding dyes and fluorescently labeled sequence-specific primers or probes are used. Real-time PCR is performed with thermal cyclers which are equipped with fluorescence detection modules. The fluorescence, which is detected after each temperature cycle, is proportional to the amount of amplified product. The cycle number at which enough amplified product accumulates to yield a detectable fluorescent signal is known as threshold cycle, or CT. The reaction has a low, or early, CT if a large amount of template, and on the other hand a high, or late, CT if a small amount of template is present at the start of the reaction. Thus, the CT value can be used to calculate the initial amount of DNA template present in a sample.

In order to analyze the diversity and richness of both the total and the potentially chitinolytic bacterial communities in the diverse lake habitats under study, barcoded 454 pyrosequencing of 16S rRNA and chitinase gene fragments were applied, respectively. 454 pyrosequencing is a high-throughput sequencing technique, which provides unprecedented sampling depth and has successfully been applied for high resolution of species diversity but also for the analysis of specific protein encoding genes (Sogin et al. 2006, Lüke and Frenzel 2011, Beier et al. 2012). During 454 pyrosequencing DNA fragments are bound to beads and are amplified by emulsion PCR resulting in millions of DNA copies per bead (Margulies et al. 2005). The beads are separated in wells of a fibre-optic slide and the DNA is sequenced by pyrophosphate sequencing (Ronaghi et al. 1998). Attached sample-specific barcoding
adapters allow for tracing the source of the DNA sequences in pooled PCR products (Meyer et al. 2008).

1.8. Objectives and outline of the thesis

This thesis was part of the interdisciplinary project “Degradation and transformation of lacustrine organic nitrogen compounds: microbiology and biogeochemistry” (SNF grants K-23K1-118111 and 200020_134798). The general objective of the project was to better understand the formation and degradation of organic nitrogen compounds in lakes and the involvement of bacteria in these transformation processes.

The focus of the biogeochemical part of the project was to study the fate of the amino compounds ASs and AAs and the proportion of bacterial biomass on the lacustrine organic matter using amino biomarkers (D-AAs and muramic acid) and bacterial cell counts. The microbial part of this thesis focused on the bacterial degradation of the amino sugar polymer chitin. The sites and significance of chitin hydrolysis were investigated in the two contrasting deep freshwater lakes, Lake Brienz and Lake Zug, which were sampled at two different seasons, spring and fall. Chitin production in the lakes was estimated from the biomasses of crustaceans and diatoms, which were assumed to be the main sources of lacustrine chitin. The identity of the potential chitinolytic bacterial consortia were determined on the sites of chitin synthesis (zooplankton), in the lake water and the sediments, where chitin is deposited. In order to investigate if the degradation of amino compounds and organic matter from primary production along the lake water columns is accompanied by bacterial community composition dynamics, the vertical shifts of the total bacterial communities were studied and related to various degradation parameters.

The thesis is structured into four main chapters which elucidate the significance of lacustrine bacteria as decomposer of complex organic matter focusing on the amino sugar polymer chitin.
Chapter 2: **Bacterial chitin hydrolysis in two lakes with contrasting trophic statuses**

In order to investigate the main sites of lacustrine chitin hydrolysis, the turnover rate of the chitin analog methylumbelliferyl-\(N,N'\)-diacetylchitobioside (MUF-DC) and the presence of chitinase genes (\(chiA\)) in zooplankton, water collected along the entire water columns, and the sediments of Lake Brienz and Lake Zug were measured. The results were linked to concentrations of chitin’s monomer GlcN detected in the diverse samples and lake habitats and to concentrations of organic carbon and nitrogen to estimate the significance of chitin as carbon and nitrogen source. Chitin production in the lakes was estimated from zooplankton (crustaceans) and diatom biomasses.

Chapter 3: **Rare bacterial community members dominate the functional trait of chitin hydrolysis in freshwater lakes**

In order to identify the chitinolytic bacterial consortia on the sites of chitin synthesis (zooplankton), in the water and its sink, i.e., the sediments, 454 pyrosequencing of 16S rRNA and \(chiA\) genes was applied on selected samples for which high \(chiA\) gene abundance was detected.

Chapter 4: **Impact of amino compounds and organic matter degradation state on the vertical structure of lacustrine bacteria**

The vertical shifts in the composition of the free-living and particle-associated bacterial communities were analyzed in the water columns of Lake Brienz and Lake Zug via the DNA fingerprinting method automated ribosomal intergenic spacer analysis (ARISA). The observed patterns were linked to physico-chemical parameters and to various parameters indicating particulate organic matter degradation state and composition. The statistical method of ordination analysis in combination with forward selection was used to extract the environmental variables with the strongest influence on the vertical bacterial community composition dynamics.
Chapter 5: Contribution of bacterial cells to lacustrine organic matter based on amino sugars and D-amino acids

In order to study the fate of particulate ASs, the concentrations of particulate GlcN, GalN, muramic acid, and mannosamine were measured along the water columns of Lake Brienz and Lake Zug. The contribution of bacterial cells to the organic carbon in the lakes were estimated using the amino biomarkers muramic acid and D-AAs. The results were compared to estimates using bacterial cell counts.
2. BACTERIAL CHITIN HYDROLYSIS IN TWO LAKES OF CONTRASTING TROPHIC STATUSES

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accepted *Applied Environmental Microbiology*


Contributions of the authors:
The paper was written by Krista Köllner and corrected by Helmut Bürgmann. The sampling was done by Dörte Carstens and Krista Köllner. Krista Köllner conducted the chitinase activity measurements, the quantitative chitinase gene detection (qPCR), analysis and interpretation of the data. Dörte Carstens did the biogeochemical analysis. Esther Keller analyzed the zooplankton and phytoplankton communities of Lake Zug. Francisco Vazquez provided technical support for all the analyses and samplings. Carsten Schubert significantly contributed to the conception of the study and data interpretation. Josef Zeyer added significant contribution to the interpretation of the data. Helmut Bürgmann significantly contributed to the conception, the design, and the interpretation of the presented study.
2.1. Abstract

Chitin, which is a biopolymer of the amino sugar glucosamine (GlcN), is highly abundant in aquatic ecosystems and its degradation is assigned a key role in the recycling of carbon and nitrogen. In order to study the significance of chitin decomposition in two temperate freshwater lakes of contrasting trophic and redox conditions, we measured the turnover rate of the chitin analog methylumbelliferyl-N,N'-diacetylchitobioside (MUF-DC) and the presence of chitinate genes (chiA) in zooplankton, water, and sediment samples. In contrast to the eutrophic and partially anoxic lake, chiA gene fragments were detectable throughout the oligotrophic water column and chiA copy numbers per ml of water were up to 15 times more abundant than in the eutrophic waters. For both lakes highest chiA abundance was found in the euphotic zone - the main habitat of zooplankton, but also the site of production of easily degradable algal chitin. The bulk of chitinate activity was measured in zooplankton samples and the sediments, where recalcitrant chitin is deposited. Both, chiA abundance and chitinate activity correlated well with organic carbon, nitrogen, and concentrations of particulate GlcN. Our findings show that chitin, although its overall contribution to total organic carbon was small (~0.01-0.1%), constituted an important microbial growth substrate in these temperate freshwater lakes, particularly where other easily degradable carbon sources were scarce.

2.2. Introduction

Chitin is a homopolymer of β-1,4-linked N-acetylated glucosamine (GlcNAc). It is a structural component of the cell wall of fungi and the exoskeleton of invertebrates but is also found in protozoa (Mulisch 1993) and algae (Herth 1978, Kapaun and Reisser 1995). Due to its wide distribution chitin is, after cellulose, the second most abundant biopolymer on earth (Kirchner 1995). The annual production and the steady state amount in the biosphere is on the order of $10^{12}$ to $10^{14}$ kg (Jeuniaux and Voss-Foucart 1991, Poulicek et al. 1998). On the basis of literature data on chitin production of arthropods, the total annual chitin production in aquatic environments was estimated to $2.8 \times 10^{10}$ kg chitin year$^{-1}$ for freshwater ecosystems and to $1.3 \times 10^{12}$ kg chitin year$^{-1}$ for marine ecosystems (Cauchie 2002). The role of chitin as a significant component of the aquatic carbon and nitrogen budget was

Not only phyto- and zooplankton and insect carcasses, but also zooplankton molting (exuviae) and excretion of fecal pellets (peritrophic membranes) contribute to the production of huge amounts of chitinous particles in the water column (Weiss et al. 1996). These chitinous particles are part of the marine or lake snow which was shown to represent a hotspot of particulate organic matter solubilization (Simon et al. 1993, Grossart and Simon 1998a, b). In the ocean, chitinolytic bacteria were found to be responsible for the hydrolysis of chitin (Zobell and Rittenberg 1937, Kirchner 1995). After adhering to the polymeric substrate, chitinolytic bacteria express a multitude of enzymes and other proteins required for its catabolism (Keyhani and Roseman 1999). The hydrolysis of the β-(1,4)-glycosidic bonds between the GlcNAc residues is accomplished by extracellular chitinases (EC 3.2.1.14) (Henrissat 1991). The end products of chitin degradation in the chitinolytic pathway are monomers and dimers of GlcNAc, which can be catabolized in the cytoplasm to fructose-6-P, acetate and NH₃ (Bassler et al. 1991, Keyhani and Roseman 1999).

Based on amino acid similarities chitinases are classified into family 18 and family 19 chitinases (Henrissat 1991). Family 19 chitinases were formerly thought to be constricted to plant origin, but have since also been found in various Streptomyces species and other bacteria (Reynolds 1984, Tang et al. 2006, Beier 2010). However, most information on bacterial diversity and distribution in diverse environments is available for family 18 group A chitinases (Svitil et al. 1997, Cottrell et al. 2000, LeCleir et al. 2004, Hobel et al. 2005, Hjort et al. 2010, Lindsay et al. 2010).

In the present study we aimed to identify the main sites of chitin hydrolysis and the significance of chitin as a bacterial substrate in two temperate freshwater lakes with contrasting trophic and redox conditions. For this purpose, we analyzed the chitinase activity and the abundance of bacterial chitinase genes (chiA) in zooplankton, water from ten different depths, and sediment samples of oligotrophic Lake Brienz (LB) and eutrophic Lake Zug (LZ). The lakes were sampled in spring and fall 2009.
2.3. **Methods**

2.3.1. **Sampling sites**

The characteristics of the study sites are listed in Table 1. LB is an oligotrophic peri-alpine lake located 70 km southeast of Bern, Switzerland. The lake is fully oxic throughout the year. The catchment of the lake is drained by the two main inflows Aare and Lütschine, which together transport an annual average of $3 \times 10^8$ kg suspended material into LB (Finger et al. 2006). Both, the hydrological regime and the suspended particle load of the river Aare, are influenced by hydropower operations. The continuous supply of suspended glacial particles, causing reduced light penetration, together with the scarcity in nutrients have led to an unusually low phytoplankton biomass (on average $< 10$ g m$^{-2}$) (Finger et al. 2007, Guthruf et al. 2009).

LZ is a eutrophic sub-alpine lake 30 km South of Zurich, Switzerland. The lake consists of two basins: A shallow (40-60 m depth) North Basin and a 200 m deep South Basin. The South Basin is meromicite with mixing depths that do not exceed 100 m. Together with the eutrophic status of LZ, this leads to seasonally anoxic conditions in a depth of 140-160 m and permanent anoxia below (Moor et al. 1996, Meckler et al. 2004).

<table>
<thead>
<tr>
<th>Property</th>
<th>Lake Brienz</th>
<th>Lake Zug</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>46°43' N, 7°58' E</td>
<td>47°7' N, 8°29' E</td>
<td></td>
</tr>
<tr>
<td>Elevation (m)</td>
<td>564</td>
<td>414</td>
<td></td>
</tr>
<tr>
<td>Surface area (km$^2$)</td>
<td>29.8</td>
<td>16</td>
<td>(Moor et al. 1996, Guthruf et al. 2009)</td>
</tr>
<tr>
<td>Maximum depth (m)</td>
<td>259</td>
<td>200</td>
<td>(Moor et al. 1996, Guthruf et al. 2009)</td>
</tr>
<tr>
<td>Volume (km$^3$)</td>
<td>5.15</td>
<td>2.0</td>
<td>(Moor et al. 1996, Guthruf et al. 2009)</td>
</tr>
<tr>
<td>Primary inflow</td>
<td>Aare, Lütschine</td>
<td>Rigiaa</td>
<td></td>
</tr>
<tr>
<td>Primary outflow</td>
<td>Aare</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean hydraulic residence time (yr)</td>
<td>2.7</td>
<td>14</td>
<td>(Moor et al. 1996, Guthruf et al. 2009)</td>
</tr>
<tr>
<td>Trophic status</td>
<td>Oligotrophic</td>
<td>Eutrophic</td>
<td></td>
</tr>
<tr>
<td>Oxygen status</td>
<td>Oxic</td>
<td>Anoxic below</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>140 - 160 m</td>
<td>(Moor et al. 1996)</td>
</tr>
</tbody>
</table>
2.3 Methods

2.3.2. Sampling water and sediments

LB was sampled mid of May and mid of September 2009 in the central part of its basin at position 46°43'18"N/7°58'27"E. LZ was sampled end of March and end of October 2009 in the South Basin at position 47°6'1"N/8°29'4"E.

Profiles of temperature, oxygen (O₂), and conductivity were taken with a CTD-profiler (Seabird SBE19, Sea-Bird Electronics, Inc., Bellevue, WA). Based on the temperature and O₂ profiles determined (Fig. 6), water was sampled from ten depths using a Niskin water sampler, filled into autoclaved 1 liter glass bottles and transported cool and dark to the laboratory. We sampled LB at 5, 10, 20, 30, 40, 70, 100, 150, 200 m, and 240 m water depth, and LZ at 5, 10, 15, 25, 60, 80, 100, 130, 170, and 190 m water depth.

Particulate organic matter (POM) from the same depths was sampled on 0.7 μm glass fiber filters (Whatman Inc., Florham Park, NJ) with in situ pumps (McLane Research Laboratories Inc., Falmouth, MA) until filters were clogged.

Sediment cores were recovered from the two sampling sites using a gravity corer (Kelts et al. 1986). The first five (spring) to seven (fall) centimeters of each core were sliced at intervals of one centimeter. Subsamples of each layer were processed for microbiological and biogeochemical analysis (see below).

2.3.3. Zoo- and phytoplankton communities

Zooplankton samples were taken with a 95 μm double closing net (Bürgi and Züllig 1983) from 0-100 m (2 replicates) and preserved in 2% formaldehyde. Phytoplankton was sampled with an integrated sampler according to Schroeder (Schröder 1969) from 0-20 m (2 replicates). Lugol-fixed phytoplankton species were counted quantitatively using the technique of Utermöhl on an inverted microscope (Utermöhl 1958). Crustacean species and their development stages were enumerated under a binocular dissecting microscope at 10x–75x. Phyto- and zooplankton biomass fresh weights were calculated from mean cell/organism dimensions of each species (Bürgi et al. 1985, Guthruf et al. 2009). For LB these analyses were carried out at the Laboratory for Water and Soil Protection of Canton of Bern.
2.3.4. *Zooplankton chitin*

The chitin biomass in LB and LZ was calculated from zooplankton biomass and body chitin content published by Cauchie (Cauchie 2002), which were 4.3% and 9.8% of dry weight for lentic branchiopoda and copepoda, respectively.

2.3.5. *Chemical analysis*

The zooplankton, water, and the sediment samples were subdivided for microbiological and chemical analyses.

Dissolved organic carbon (DOC) and dissolved nitrogen (DN) concentrations in lake water were determined after filtration through a 0.2 μm Supor membrane filter.
2.3 Methods

(Pall Corporation, Port Washington, NY). DOC and total organic carbon (TOC) were measured by high temperature catalytic oxidation (720°C) with a Shimadzu TOC-V CPH (Shimadzu Scientific Instruments, Kyoto, Japan). The total nitrogen (TN) content and DN were determined with a Shimadzu TOC-V CPH / TNM1.

Phosphate (PO$_4^{3-}$) concentrations were determined following filtration through 0.45 μm cellulose acetate filters (Schleicher & Schuell GmbH, Dassel, Germany). PO$_4^{3-}$ was determined photometrically with the molybdenum blue method according to Vogler (Vogler 1965).

Concentrations of GlcN in zooplankton samples, in POM, and in the sediments were measured with a slightly modified method after Zhang and Amelung (Zhang and Amelung 1996) with a derivatization step after Guerrant and Moss (Guerrant and Moss 1984), and myo-inositol (Aldrich) as internal standard. Filters were treated with 10 ml of 6 M HCl for 10 h at 100°C, which should ensure complete hydrolysis of biopolymers of GlcN, including chitin and peptidoglycan, in which it occurs in its N-acetylated form (GlcNAc). Hydrolysis causes deacetylation and, thus, concentrations of GlcN are the sum of both forms, GlcN and GlcNAc. The gas chromatography (GC) system was equipped with a flame ionization detector (HRGC 5160, Carlo Erba Instruments, Milan, Italy), a split-splitless injector and a VF-5 MS column (60 m, 0.25 mm inner diameter and 0.25 μm film thickness). The injector temperature was 250°C and the temperature of the detector was 300°C. Hydrogen was used as carrier gas with a flow rate of 2 ml min$^{-1}$. The temperature profile was: 120°C to 200°C at 20°C min$^{-1}$, 200°C to 250°C at 2°C min$^{-1}$, 250°C to 270°C at 20°C min$^{-1}$ held 10 min at 270°C. A GlcN standard (D-glucosamine, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was also derivatized and used for quantification.

2.3.6. Chitinase activity

Chitinase activity was determined on water, sediment, and zooplankton sampled in fall 2009 using the chitin substrate analog methylumbelliferyl-N,N’-diacetetylchitobioside (MUF-DC, Sigma-Aldrich) according to (LeCleir and Hollibaugh 2006) and (Kirchman and White 1999) with slight modifications. Referring to the manufacturer’s instructions, MUF-DC was dissolved in 100% dimethyl formamide (DMF) to a final concentration of 5 mM (stock solution).
In a preliminary test, the effect of substrate concentration was determined by incubating LZ surface waters and mixed LZ sediment samples (0-5 cm) with six different MUF-DC concentrations (1, 5, 10, 50, 100, and 300 µM) at 20°C and 4°C, respectively. In addition, we tested DMF for inhibitory effects on *Streptomyces griseus* chitinase (Sigma). DMF was found to linearly inhibit activity, up to 80% at 6% DMF, the concentration in the highest MUF-DC concentration (300 µM) used in the assay. Therefore, we adjusted the concentration of DMF for all assays to 6%.

Water samples were filled into 50 ml centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) and mixed with formalin to a final concentration of 0.25% to prevent microbial growth (Hood 1991). The influence of 0.25% formalin on the fluorescence of released MUF and on the activity of *Streptomyces griseus* chitinase was tested in preliminary experiments. No significant effects were found. Formalin-fixed water samples were stored cold and dark until assayed (within 4 h after sample collection).

Sediment samples (0.5 g) and 1.48 ml of autoclaved, 0.2 µm-filtered and formalin-treated (0.25%) lake water were well mixed and assayed within 12 h of sample collection.

Water and sediment samples were amended with aliquots of the MUF-DC stock solution and incubated at 4 and 20°C, which corresponds to the minimum and maximum temperature measured in the water column of both lakes over a year. As *in situ* temperatures for different samples differ from the incubation temperatures (Fig. 6) and sediments were analyzed as slurries, our data (Fig. 9 and Fig. 10) are potential chitinase activity rates. After incubation for between 1 and 3 h, the reactions were stopped in aliquots of 100 and 150 µl of water and sediment-in-water-suspension by adding 10 and 15 µl of ammonium glycine buffer (pH 10.5) (Daniels and Glew 1984), respectively. Fluorescence of free methylumbelliferone (MUF) was measured in the water samples and sediment supernatants at 360 nm excitation and 460 nm emission using a Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Samples were shaken (300 rpm) between measurements.

Zooplankton was killed by 3% hydrochloric acid and washed with autoclaved and 0.2 µm-filtered lake water. 0.05 g of zooplankton were suspended in 2 ml 0.25% formalin-treated, autoclaved, and 0.2 µm-filtered lake water. After adding an aliquot of the MUF-DC stock solution, samples were incubated and processed as described.
for the sediments. Sediments were measured repeatedly up to 11 h, zooplankton up to 9 h, and water up to 100 h.

Positive controls were assayed in autoclaved, 0.2 µm-filtered and formalin-treated (0.25%) lake water and in autoclaved sediments containing aliquots of MUF-DC stock solution and *Streptomyces griseus* chitinase. In addition negative controls without *Streptomyces griseus* chitinase were run to test for abiotic degradation of MUF-DC.

### 2.3.7 DNA extraction

Autoclaved glass bottles (1 liter) were filled with water samples and transported on ice and in the dark to the laboratory. About five liters of water from each sampled depth were filtered through a 5 µm isopore membrane filter (Millipore, Billerica, MA) and a 0.2 µm polycarbonate filter (Whatman) each 142 mm in diameter, connected in series. The filters were frozen in liquid nitrogen immediately after filtration and stored at -80°C until DNA extraction. For extraction, a filter segment (1/4) was cut into small pieces using sterile scissors and mixed with 0.2 g of glass beads (0.1 g of 106 µm and 0.1 g of 150-212 µm glass beads, Sigma-Aldrich) in a 2 ml screw cap tube (Brand GmbH & Co KG, Wertheim, Germany). 1.4 ml of ice-cold extraction buffer (Hönerlage et al. 1995) were added. Cells were disrupted in a FastPrep-24 bead-beating system (MP Biomedicals, Solon, OH) by beating twice for 40 s at 4 m s⁻¹, placing tubes on ice in between. Bead-beating was followed by a freeze-thaw cycle in liquid nitrogen. The supernatant was treated with 50 µg ml⁻¹ RNase A (Sigma-Aldrich) for 30 min at 37°C and extracted with an equal volume phenol-chloroform-isoamylalcohol (25:24:1) (pH 8) (Sigma-Aldrich). After precipitation with one volume of isopropanol, the pelleted DNA was dissolved in Tris-EDTA (pH 8) buffer and stored at -80°C.

For extraction of sediment and zooplankton 0.5 g and 0.05 g sample, respectively, were mixed with ice-cold 1.4 ml nucleic acid extraction buffer (Hönerlage et al. 1995). Samples were frozen in liquid nitrogen and stored at -80°C until DNA extraction. After thawing 0.25 g sterile 0.1 mm Zirconia beads (Biospec Products Inc., Bartlesville, OK) were added and samples were processed on a vortex adaptor (MoBio Laboratories, Inc., Carlsbad, CA) for 1 min at maximum speed. DNA extraction was performed as for the water filters.
The quality of DNA extracts was checked by agarose gel (1%) electrophoresis. Extracted DNA was quantified by fluorescence spectroscopy using the Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes, Eugene, OR) and a Synergy HT microplate reader (Bio-Tek Instruments).

The reproducibility of the applied DNA extraction protocols was tested on quadruplicate samples of sediment slurries and on four ¼ segments of 0.2 µm polycarbonate filters, one for each lake.

2.3.8. Amplification and quantification of chitinase gene fragments

We used the primer pair chif2 (GACGGCATCGACATCGATTGG) and chir (CSGTCCAGCGCGSCRTA), which were reported to target chitinase family 18 group A (chiA) gene fragments from a broad range of chitinolytic bacteria previously (Xiao et al. 2005). The performance and specificity of chiA PCR primers were tested on spring water samples (LB 10 m, LB 240 m, LZ 10 m, and LZ 190 m).

Each PCR reaction (20 µl) contained: 10 µl 2x Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City CA), 0.4 µl of each primer (10 µM, Microsynth, Balgach, Switzerland), 2 µl BSA (10 mg ml⁻¹, Sigma-Aldrich) and 5 µl template diluted in nuclease free water (Qiagen GmbH, Hilden, Germany). Amplification was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) with PCR conditions consisting of an initial denaturation step at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 60 s, and extension at 72°C for 30 s followed by melting-curve analysis.

PCR resulted in products yielding a single sharp band of the expected size of ~430 bp in gel electrophoresis (data not shown). To evaluate the specificity of the chiA PCR primers, we constructed clone libraries from the spring water samples. PCR products of chitinase gene fragments from samples were cloned into the pGEM-T vector by using the pGEM-T Easy Vector System according to the instructions of the manufacturer (Promega). Screening of the clone libraries constructed from these samples resulted in 15 different RFLP types among 71 screened plasmids. We sequenced 1-5 clones of each RFLP type, 31 clones in total (sequencing by Microsynth AG, Balgach, Switzerland). All sequences could be assigned to chitinases, indicating a high specificity of the PCR protocol (unpublished data).

In preliminary experiments, the PCR amplification was assayed in four-fold serial dilutions of total DNA extracts in nuclease free water (Qiagen) to determine the
effect of inhibiting contaminants. For instance, according to the resulting copy numbers, we assayed lake sediments in 64-fold dilutions, as they gave comparable, but slightly higher (~13%) chiA copy numbers compared to 16-fold diluted templates while results dropped off significantly for the 256-fold dilution (~40%). Four-fold dilution of zooplankton samples, 16-fold, and 64-fold dilutions of water and sediment samples, respectively, were applied in the final analysis. Therefore, different amounts of genomic DNA template were used in qPCR, i.e., less than 5 ng DNA for LB samples and ~10-20 ng DNA for LZ samples. Additionally, results of quadruplicate analysis of DNA extraction yields showed high standard deviations for water filters, up to 31%. For sediment slurries the standard deviation was 6%.

2.4. Results

2.4.1. Biogeochemistry of lake water columns

(i) Oxygen. The temperature, O₂, and conductivity profiles for both lakes sampled in spring and fall are shown in Fig. 6. In both seasons the water column of LB was fully oxic. For LZ anoxic conditions were measured below 130 m (O₂ < 0.1 mg liter⁻¹), a shallower depth than reported previously (Moor et al. 1996, Meckler et al. 2004).

(ii) Organic carbon. In LZ waters, the TOC concentrations were roughly three to four times higher compared to LB waters (Fig. 7A) and ranged from 1.86 to 2.54 mg C liter⁻¹ (error of measurement is 0.20 mg C liter⁻¹). The DOC values ranged between 1.80 ± 0.20 mg C liter⁻¹ (March 2009; 170 m) and 2.40 ± 0.20 mg C liter⁻¹ (October 2009; 5 m). For LB the DOC concentrations were below or close to the detection limit of 0.50 mg C liter⁻¹ in both seasons.

(iii) Nitrogen. The TN and the DN concentrations were below the detection limit of 0.50 mg N liter⁻¹, except for LZ waters in spring (data not shown).

(iv) Phosphate. The $PO_4^{3-}$ concentrations were below 5 µg P liter⁻¹ for all sampled water depths of LB, with the exception of the 5 m and the 20 m surface water depths sampled in September 2009 (Fig. 7C). For LZ the $PO_4^{3-}$ concentrations increased with water depth. The concentrations ranged from 3.8 ± 0.5 µg P liter⁻¹ (October 2009; 15 m) to 217 ± 0.5 µg P liter⁻¹ (October 2009; 190 m).

(v) Glucosamine. For both lakes, concentrations of particulate GlcN were highest in the euphotic zone and decreased with depth (Fig. 7D). The highest value of
70.6 nmol liter\(^{-1}\) was measured in the 5 m water depth of LZ sampled in March 2009. For LB, the GlcN concentrations were roughly one order of magnitude lower compared to LZ.

Fig. 7. Water column properties of Lake Brienz (LB) sampled in May and September 2009 and of Lake Zug (LZ) sampled in March and October 2009 as a function of depth. (A) TOC ± 0.2 mg C liter\(^{-1}\), (B) ± 0.02 mg N liter\(^{-1}\), (C) ± 0.5 µg P liter\(^{-1}\), errors given are standard deviations of measurement, (d) particulate glucosamine (GlcN) in nmol liter\(^{-1}\) of water. Standard deviation of GlcN measurement was below 10%. Bold dashed lines mark border between oxic and anoxic water body of LZ.
2.4.2. Biogeochemistry of sediment profiles

For LB sediments, the TOC, TN, and GlcN contents were always lowest for the 1-2 cm layer, in which the sediment gets anoxic (Müller et al. 2007), and increased again with depth (Table 2). Biogeochemical parameters in LZ sediment (Table 3) were roughly one order of magnitude higher compared to LB sediments - with the exception of the 6-7 cm layer sampled in fall, which showed similar TOC, TN, and GlcN contents in both lakes.

<table>
<thead>
<tr>
<th>Table 2. Biogeochemistry of sediments of Lake Brienz (LB) sampled in May and September 2009</th>
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<tr>
<td>Core LB, May 2009</td>
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<tr>
<td>Depth (cm)</td>
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<tr>
<td>0-1</td>
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<td>1-2</td>
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<td>2-3</td>
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<td>4-5</td>
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<td>5-6</td>
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Error of measurement (standard deviation) of one sample: TOC ± 0.1%, TN ± 0.02%, GlcN ± 10%.

<table>
<thead>
<tr>
<th>Table 3. Biogeochemistry of sediments of Lake Zug (LZ) sampled in March and October 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core LZ, March 2009</td>
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<tr>
<td>Depth (cm)</td>
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Error of measurement (standard deviation) of one sample: TOC ± 0.1%, TN ± 0.02%, GlcN ± 10%.
2.4.3. Zoo- and phytoplankton community composition

The phytoplankton communities in both lakes was composed of chrysophytes (golden algae), diatoms, cryptophytes, dinoflagellates, chlorophytes (green algae), haptophytes (only in LB in May 2009), euglenoids (only in LZ in October 2009), and cyanobacteria. The phytoplankton biomass was dominated by diatoms except for LZ in March 2009 when the predominant organisms were cyanobacteria (60%). Diatoms accounted for 40% (May 2009) and 66% (September 2009) of the biomass in LB and for 16% (March 2009) and 71% in LZ (October 2009).

In both lakes the predominant zooplankton species were cladocerans (*Daphnia* sp., *Diaphanosoma brachyurum*, *Leptodora kindtii*, *Bosmina* sp. (only in LZ in March 2009)) and calanoid copepods (*Diaptomidae*). The vast majority of the zooplankton biomass consisted of copepods. For the spring sampling campaign they accounted for over 99% of the zooplankton biomass in both lakes and for the fall sampling campaign for 61% and 80% in LB and LZ, respectively.

2.4.4. Zoo and phytoplankton biomass

In March/May 2009, the zoo- and phytoplankton biomasses contributed equally to the total plankton biomass in both lakes (Fig. 8). In LZ, the plankton biomass (25.9 g m$^{-2}$) was more than double that of LB (11.7 g m$^{-2}$). In October 2009, the vast majority of the LZ plankton biomass (33.9 g m$^{-2}$) was phytoplankton (> 90%). The late fall phytoplankton bloom is in agreement with chlorophyll $a$ measurements from the same year (Environmental Agency of Canton Zug, unpublished data). The low proportion of zooplankton biomass also goes along with the predominance of large grazing-resistant forms of diatoms such as *Asterionella formosa* and *Fragilaria crotonensis*, and the scarcity of food sources for zooplankton like small algae and cyanobacteria (data not shown). In contrast, the zooplankton biomass from LB collected in September 2009 accounted for 72% of the total plankton biomass of 28.1 g m$^{-2}$. Judging from LB plankton monitoring data for the year 2009 (Environmental Agency of Canton Bern, unpublished data), the zoo- and phytoplankton biomasses generally were approximately equal with the exception of January and late summer (July-September), when the zooplankton biomass was significantly greater (up to 74% of the total plankton biomass).
2.4 Results

2.4.5. Zooplankton chitin

In March / May 2009, chitin estimated from zooplankton abundances was 134 mg m$^{-2}$ for LZ and therefore more than 2 times higher than in LB (55.3 mg m$^{-2}$) (Fig. 8). In contrast, in fall, zooplankton chitin in LB (154 mg m$^{-2}$) was about 8 times higher than in LZ (18.7 mg m$^{-2}$).

![Fig. 8. Zoo - and phytoplankton fresh biomass, zooplankton chitin estimate, and particulate glucosamine (GlcN) summarized over 0-100 m water depths for Lake Brienz (LB) sampled in May and September 2009 and for Lake Zug (LZ) sampled in March and October 2009.](image)

2.4.6. Zooplankton chitin biomass compared to TOC and GlcN concentrations

For the spring sampling campaign, relating the chitin estimates from the zooplankton to the carbon pool, zooplankton chitin contributed 0.04% of the TOC integrated over the upper 100 m of the water column of LB (63.5 g C m$^{-2}$) and 0.03% of the TOC pool of LZ (205 g C m$^{-2}$). For the fall sampling campaign, the contribution of zooplankton chitin to the TOC was almost 30 times higher in LB (0.1270%) compared to LZ (0.0045%).

In LZ, the particulate GlcN concentration integrated over the 0-100 m water column was about 2-fold (March 2009) and 6-fold (October 2009) higher compared to the zooplankton chitin biomass (Fig. 8). In LB, it was as high as the zooplankton chitin biomass in May 2009 but it was only one sixth of zooplankton chitin biomass in September 2009. This discrepancy could be caused by the higher inorganic glacial particle load from the inflows in the second half of the year (Finger et al. 2006, Finger...
et al. 2007), which caused a higher turbidity in the surface waters (AWA Amt für Wasser und Abfall). As a consequence, the primary production maximum was shifted to a lower water depth of 2.5 m compared to May 2009 when it was observed at a water depth of 5 m (Carstens et al. 2012). Since the shallowest in situ pumping was performed at 5 m, the fall sampling probably missed a significant proportion or even the maximum of the zooplankton biomass in the surface waters of LB.

2.4.7. Zooplankton chemistry

The GlcN content of zooplankton sampled in fall accounted for 69.6 ± 4.5 and for 94.8 ± 7.1 µmol g⁻¹ dry wt in LB and LZ, respectively, and was, therefore, about one third higher in the zooplankton of LZ. The results for the TOC, TN, and GlcN content of zooplankton samples are summarized in Table 4.

| Table 4. Zooplankton chemistry, chitinase activity, and chiA copies on zooplankton samples
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<thead>
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<tbody>
<tr>
<td>Site</td>
</tr>
<tr>
<td>LB</td>
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<tr>
<td>LZ</td>
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</table>

The errors given are standard deviations of triplicate measurements of one sample. MUF, fluorescent methylumbelliferone released after the hydrolysis of the chitin substrate analog methylumbelliferyl-N,N’-diacetylchitobioside.

2.4.8. Chitinase activity

The effect of the substrate concentration on chitinase activity was tested in LZ surface waters and mixed sediment slurries. For the sediment samples, MUF-DC turnover was found highest (2.63 ± 0.22 nmol h⁻¹ g dry sediment⁻¹) at a substrate concentration of 50 µM (Fig. 9) and dropped at 100 and 300 µM. For the water samples the chitinase activity was below the limit of detection for all substrate concentrations even after 4 days of incubation. For negative controls, no turnover rates could be detected, which implies that only biotic degradation of MUF-DC was detected during the incubation.
We assayed the sediments and zooplankton from both lakes sampled in fall 2009 with 50 µM MUF-DC at 4°C and 20°C. MUF-DC turnover rates at 20°C were roughly one-third higher than at 4°C for the zooplankton samples of both lakes and up to 3- and 8-fold higher for LB and LZ sediments, respectively. For reasons of simplicity, only the data for 4°C are discussed and shown in Table 4 and Fig. 10.

(i) Zooplankton. Chitinase activity on LZ zooplankton was about double that measured for LB zooplankton on a dry weight basis (Table 4).

(ii) Sediment. Chitinase activity (at 4°C) in LB sediments ranged from 0.08 (1-2 cm) to 0.69 nmol MUF h⁻¹ g dry wt⁻¹ (6-7 cm) and in LZ sediments from 0.59 (5-6 cm) to 5.10 nmol MUF h⁻¹ g dry wt⁻¹ (0-1 cm) (Fig. 10). Comparing the two lake systems, LZ’s chitinase activity per gram dry sediment was up to 40 times higher than that measured in LB sediments, but decreased with depth and converged on LB’s values in the 5-6 and 6-7 cm layer (Fig. 10). However, normalized to the GlcN concentrations, the chitinase activities were in the same range for both lakes, with no clear depth-related trend (data not shown).

Normalized to the TOC, TN, and GlcN contents, the chitinase activities were in the same order of magnitude as the values measured for zooplankton (data not shown).
Bacterial chitin hydrolysis in two lakes of contrasting trophic statuses

Fig. 10. Chitinase activities in the upper 7 centimeter of Lake Brienz (LB) and Lake Zug (LZ) sediment relative to sediment dry weight in fall 2009 at 4°C. MUF, fluorescent methylumbelliferone released after the hydrolysis of the chitin substrate analog methylumbelliferyl-N,N’-diacetylchitobioside. The error bars represent standard deviations of triplicate measurements of one sample.

2.4.9. Chitinase gene copies

(i) Water. For the 0.2-5 µm water fractions of LZ, specific chiA fragments could be amplified only for the 5 m and the 10 m water depths sampled in spring and for the 5 m to 25 m water depths sampled in fall (Fig. 11). In comparison, the chiA gene copies detected per ml of water were 2 to 15 times higher in the surface waters of LB, with the exception of the 10 m water depth of LZ in October 2009 (272 ± 17 chiA copies ml⁻¹). The maximum chiA concentration in LB waters (340 ± 7 chiA copies ml⁻¹) was measured in the 5 m water depth in May 2009. In LB, the chiA gene was detected at all depths. For the ≥ 5 µm fractions, we got no specific chiA signals for LB. ChiA was detected in the surface water depths of LZ in this fraction. However, concentrations were up to 20 times lower than for the 0.2-5 µm fractions (data not shown).
Fig. 11. *chiA* gene copy number in the water columns of Lake Brienz (LB) and Lake Zug (LZ). (A) *chiA* copies per ml of water. (B) *chiA* copies per ng extracted DNA. (C) *chiA* copies per pmol GlcN. (D) *chiA* copies per µg TOC. The error bars represent standard deviations of triplicate measurements of one sample.

(ii) Sediment. In LB sediment, the lowest *chiA* content was measured in the 1-2 cm layer, increasing again with depth below this layer, as observed for the chitinase activity (Fig. 10 and Fig. 12A). In May 2009, the highest *chiA* content of $87.0 \pm 9.2$ *chiA* copies mg dry wt$^{-1}$ was measured in the 0-1 cm sediment layer and in September 2009 in the 6-7 cm layer ($363 \pm 49$ *chiA* copies mg dry wt$^{-1}$). The *chiA* content in the 0-1 cm layer of LZ sediment was about 2 (October 2009) to 10 times (March 2009) higher than that of LB on a dry weight basis. Normalized to the GlcN (Fig. 12C), TOC (Fig. 12D), and TN (data not shown) contents of the sediment, the *chiA* copy numbers were higher in LB, with few exceptions.

Since the efficiency of any nucleic acid extraction method from environmental samples is less than 100% and we did not use an internal standard to correct for this, the absolute copy numbers of *chiA* were likely underestimated. Variable extraction efficiencies may affect comparisons between different sample types. Therefore, *chiA* copy numbers normalized to the amount of DNA used in the PCR are shown in Fig.
11B and Fig. 12B. For the water column of LB, chiA gene copy numbers per ng DNA exceeded the numbers detected for the surface waters (Fig. 11B). In the sediments, chiA gene copy numbers per ng DNA were higher for all sediment layers of LB than for the sediments of LZ (Fig. 12B).

(iii) Zooplankton. We determined the chiA gene copy number on zooplankton samples, considering them as a main source of chitin in our lake ecosystem and therefore as hotspot of bacterial chitin hydrolysis. The number of chiA copies detected in LZ zooplankton samples was more than 3-fold higher than in LB zooplankton samples on a dry weight basis (Table 4). Normalized to the amount of DNA used in the PCR, it was 10-fold higher.

Normalized to the GlcN concentrations, the chiA copy numbers associated with zooplankton were approximately in the same order of magnitude as the chiA copies in the sediment of LZ but up to 10-fold lower than the values detected in the
sediment from LB (Fig. 12C). Compared to the results for the water columns, the chiA concentrations normalized to GlcN were 100- to 1000-fold lower in zooplankton samples from LZ and LB, respectively (Fig. 11C).

2.4.10. Correlations between chitinase activity, chiA abundance, and biogeochemical parameters

(i) Water. For both sampling campaigns, the chiA copies detected in the water column of LB and the GlcN concentration were highly significantly correlated (P < 0.01; n = 10) (Table 5).

(ii) Sediment. For both lake sediments, the chiA content and the chitinase activity correlated highly significantly (P < 0.01; n = 7) (Table 5). For the LB core sampled in fall, significant correlations were found between the chiA copy number and the GlcN, TOC, and TN contents. In spring, these correlations were not significant, except for the correlation between chiA copy number and GlcN. Chitinase activity and GlcN, TOC, and TN concentrations also correlated significantly for the fall cores from both lakes, but in LZ, the correlation was not as highly significant as in LB sediments.
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<tbody>
<tr>
<td>Chitinase gene copy no.</td>
<td>0.84</td>
<td>0.83</td>
<td>0.69</td>
<td>0.21</td>
<td>0.22</td>
<td>0.48</td>
<td>0.85</td>
<td>0.72</td>
</tr>
<tr>
<td>Chitinase activity at 4°C</td>
<td>0.58</td>
<td>0.50</td>
<td>0.85</td>
<td>0.84</td>
<td>0.72</td>
<td>0.47</td>
<td>0.72</td>
<td>0.72</td>
</tr>
<tr>
<td>GlcN (μmol g dry wt⁻¹)</td>
<td>0.90</td>
<td>0.86</td>
<td>0.69</td>
<td>0.48</td>
<td>0.48</td>
<td>0.85</td>
<td>0.72</td>
<td>0.72</td>
</tr>
<tr>
<td>TOC (% dry wt)</td>
<td>0.20</td>
<td>0.14</td>
<td>0.22</td>
<td>0.21</td>
<td>0.22</td>
<td>0.48</td>
<td>0.85</td>
<td>0.72</td>
</tr>
<tr>
<td>TN (% dry wt)</td>
<td>0.84</td>
<td>0.91</td>
<td>0.82</td>
<td>0.68</td>
<td>0.79</td>
<td>0.70</td>
<td>0.96</td>
<td>0.64</td>
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</table>

**P < 0.05.**

**P < 0.01.**
2.5 Discussion

2.5.1 Sources of chitin and glucosamine in freshwater lakes

Chitin was reported to be produced in high amounts in aquatic ecosystems previously (Poulicek et al. 1998, Cauchie 2002). The mean chitin standing biomass from marine planktonic crustaceans (copepods, cladocerans, and decapod larvae) was calculated to 26.3 mg m\(^{-2}\) (Jeuniaux and Voss-Foucart 1991), which is in the same order of magnitude as the zooplankton chitin biomass we estimated for our two different lacustrine ecosystems. Our results also agree with the results from a study on chitin dynamics in a mesotrophic bog and a hypereutrophic lake, in which the chitin biomass (from crustaceans) fluctuated between 2 and 200 mg m\(^{-2}\) (Miyamoto et al. 1991). The available data thus points to similar chitin biomass in marine and lacustrine ecosystems, with considerable local and seasonal variability.

However, zooplankton is not the only chitin source in an aquatic ecosystem. It is known that chitin is also produced by protozoa, fungi, and algae, especially by diatoms (Herth 1978, Bartnicki-Garcia and Lippman 1982, Mulisch 1993). Diatom chitin, known as chitan, can amount to a significant constituent of the cellular biomass. For the diatom *Thalassiosira fluviatilis*, e.g., chitan was found to represent 31–38% of the total cell mass (McLachlan et al. 1965). In contrast, Smucker reported only 7% and found significant differences between various diatom species (ranging from 2 to 10% of dry wt) (Smucker 1991). However, he attributed this discrepancy also to chitan losses due to the extraction method that had been used. To our knowledge, chitin content estimates for diverse algal species are not available in the literature, and a calculation of phytoplankton chitin, analogous to the one provided for zooplankton chitin, is thus currently not feasible. As a rough estimate, assuming a chitan content between 5% and 30% of dry diatom biomass and a mean dry weight of 300 pg per diatom cell (Reynolds 1984), diatom chitin would constitute 102 to 610 mg m\(^{-2}\) for LB in May 2009, 177 to 1061 mg m\(^{-2}\) for LB in September 2009, 261 to 1568 mg m\(^{-2}\) for LZ in March 2009, and 503 to 3015 mg m\(^{-2}\) for LZ in October 2009. Thus, significant amounts of chitin, exceeding the zooplankton contribution, may have been produced by this source. This may also explain our observations in LZ, where the particulate GlcN pool (0–100 m) was indeed found to be higher than the zooplankton chitin (Fig. 8).
GlcN is not only the main constituent of the biopolymer chitin, but together with muramic acid (MurA), also forms the disaccharide backbone of the bacterial cell wall polymer peptidoglycan. Based on measurements of MurA concentrations Carstens et al. determined the contribution of bacterial cells to the particulate GlcN (Carstens et al. 2012). Bacteria-derived GlcN accounted for up to 26% and 34% of total GlcN in the euphotic zone of LZ and LB, respectively. Compared to the water column of LZ the proportions of bacterial GlcN were higher in the water column of LB with a maximum of 94% at 200 m sampled in fall 2009.

2.5.2. Chitinolytic activity and populations in freshwater lakes

Several studies on chitinase activity in aquatic environments have been published, mainly for marine and estuarine water and sediments, which applied a wide range of substrate concentrations, ranging from 20 nM to 5 mM (Hoppe 1983, Hood 1991, Smith et al. 1992, Vrba et al. 1996, Kirchman and White 1999, Bowman et al. 2003, Mudryk and Skórczewski 2004). This makes the determination of substrate saturation curves for the environment under study crucial. We found an optimum substrate concentration of 50 µM for the lake sediments, whereas no chitinase activity could be measured in the water at any substrate concentration. Using a comparable approach (using 20 µM MUF-GlcNAc), Martinez et al. (1996) could not detect any chitinase activity in seawater (Martinez et al. 1996). In contrast, in alkaline hypersaline Mono Lake turnover rates for 10 µM MUF-DC in water and sediment samples were 1000-fold higher compared to the chitinase activities of the sediments analyzed in the present study (LeCleir and Hollibaugh 2006). However, Mono Lake is an environment extremely rich in chitin from shrimp exuvia and carcasses. The lack of detection in the water samples of our lake ecosystems indicates that chitinase activity was mostly associated with particles. In aquatic environments aggregates are known as hotspots of exoenzymatic activities (Smith et al. 1992, Grossart and Simon 1998a). Chitinase activity, in particular, was reported to be mainly associated with particulate fractions previously, e.g., they were found associated with the > 3 µm or the 2-100 µm particle size class (Hoppe et al. 1998, Richardot et al. 1999). Similarly, we found significant activity in the water only on the zooplankton samples, i.e. the > 95 µm fraction. Particle-associated microbes have the advantage to benefit directly from the soluble oligomers produced by chitin hydrolysis. However, planktonic bacteria can
also profit from chitin hydrolysis products, which has been shown recently for members of planktonic freshwater *Actinobacteria* (Beier and Bertilsson 2011).

By *chiA*-specific quantitative PCR we could confirm the presence of chitinolytic bacteria in zooplankton, sediment, and the 0.2-5 µm water fractions. The *chiA* abundance normalized to any parameter (DNA, GlcN, TOC) that was associated with the 0.2-5 µm water fraction was significantly higher than what was detected in the zooplankton samples, which we had assumed to contain high concentrations of chitinolytic bacteria. The access to zooplankton chitin (α-chitin) is probably hindered by cross-linked structural components such as glycans and proteins. These have to be degraded by different microbial communities initializing the decomposition of zooplankton carcasses (Gooday 1990, Tang et al. 2006, Tang et al. 2009). However, we did not analyze the fraction of eukaryotic DNA in the DNA extracts to normalize the results to only bacterial DNA, which could change the described ratios.

The bacterial degradation of chitin is known to be highly regulated (33). Therefore, the detection of bacterial *chiA* gene copy numbers only indicates the presence of bacteria capable of chitin degradation and is not a direct measure for active chitin hydrolysis, i.e., chitinase activity. However, in the present study *chiA* gene abundance in sediments was highly correlated to chitinase activity, and the increased abundance of the *chiA* gene in the water column of LB, where chitin had a higher contribution to the carbon pool, also indicates a relationship of gene abundance and chitin degradation. The higher prominence of chitin as a bacterial substrate in oligotrophic compared to eutrophic lakes has also been shown in culture-dependent analyses of chitinolytic bacteria in Polish lakes (Donderski 1984, Donderski and Brzezinska 2003).

In conclusion, significant correlations between *chiA* gene abundance, chitinase activity and biogeochemical data evidenced the contribution of chitin to the carbon and nitrogen budget in the lake sediments, in particular for the oligotrophic system, LB (Table 5). We therefore assign chitin a role as a significant microbial growth substrate in temperate freshwater lakes, especially where other easily degradable carbon sources are scarce.
3. RARE BACTERIAL COMMUNITY MEMBERS DOMINATE THE FUNCTIONAL TRAIT OF CHITIN HYDROLYSIS IN FRESHWATER LAKES

Krista E. Köllner and Helmut Bürgmann

Contributions of the authors:
The manuscript was written by Krista Köllner and corrected by Helmut Bürgmann. Krista Köllner conducted the DNA extraction and the amplification of 16S rRNA gene and chitinase gene fragments subjected to 454 pyrosequencing. Krista Köllner performed the analysis and interpretation of the data in collaboration with Helmut Bürgmann.
3.1. Abstract

Currently, little is known about the identity and ecology of freshwater chitinolytic bacteria, which play a key role in the hydrolysis of chitin. In a recent study in two freshwater lakes with contrasting trophic status, we found remarkably high chiA gene copy numbers in the oligotrophic compared to the eutrophic water column. However, for both lakes the chitinolytic activity was only detected in zooplankton and sediment samples. For the present study, we characterized potentially chitinolytic bacteria by 454 pyrosequencing of the chiA diversity in zooplankton, water, and surficial sediments of both lakes. The abundance of the potentially chitinolytic bacteria was related to the bacterial community composition analyzed by 454 pyrosequencing of the 16S rRNA gene. The dominant pelagic bacteria in both lakes were Actinobacteria. However, except for one water sample, chitinases associated with actinobacterial chitinases were only abundant in the sediments and the zooplankton sample of the oligotrophic lake. The predominant chitinase sequence in the water of this lake matched that of Stenotrophomonas maltophilia. As it was not abundant in the particulate samples, Stenotrophomonas maltophilia may rather be involved in scavenging chitin hydrolysis products released to the water column. In the eutrophic lake a single phylogenetically unidentified chitinase lineage was dominant in all three habitats. Other abundant sequences were associated with chitinases of the β-Proteobacteria and Actinobacteria. Although, the total diversity of the detected chitinase sequences was striking, the bulk of chitin hydrolysis appeared to be associated with a few dominant species that varied by environment.

3.2. Introduction

Chitin is synthesized as a structural component by many organisms, for instance by fungi, arthropods, and algae (Gooday 1990). Thus, chitin is one of the most abundant biopolymers on earth (Gooday 1990). In aquatic ecosystems, chitin synthesis is estimated at 10^{10} to 10^{12} kg chitin year^{-1} (Cauchie 2002). Chitin is a highly insoluble polymer of N-acetylglucosamine and prior to assimilation, it has to be hydrolyzed to soluble oligomers and dimers of N-acetylglucosamine (Keyhani and Roseman 1999). In aquatic ecosystems, the ability to hydrolyze chitin is mainly
attributed to bacteria and accomplished by chitinases (EC 3.2.1.14) (Zobell and Rittenberg 1937, Boyer 1994).

Bacterial chitinases are mainly family 18 glycoside hydrolases (Henrissat 1999). However, family 19 glycoside hydrolases were also detected for *Streptomycetes* spp. and a couple of other bacterial species (Watanabe et al. 1999, Kong et al. 2001, Honda et al. 2008). Family 18 chitinases are classified into groups A, B, and C according to amino acid sequence similarities within their catalytic domains (Watanabe et al. 1993). Bacterial chitinases are highly diverse and the apparent phylogeny of chitinase gene sequences was found not to correspond to the 16S rRNA gene based phylogeny (Cottrell et al. 2000, Xiao et al. 2005). This discrepancy is attributed to lateral gene transfer between organisms, which is probably also the reason for the detection of multiple chitinase genes in a single species (Saito et al. 1999, Cottrell et al. 2000, Karlsson and Stenlid 2009). The expression of multiple chitinases from different chitinase genes empowers an organism to hydrolyze chitin efficiently and the synergistic properties of multiple chitinases have been described elsewhere (Saito et al. 1999, Suzuki et al. 2002, Orikoshi et al. 2005). The ability to express multiple chitinases is specifically relevant given the structural diversity of chitin (α-, β-, γ-arrangement of N-acetylglucosamine strands, variation in the degree of deacetylation, cross-linking to other structural components such as glucans and proteins (Gooday 1990)) and guarantees the efficient utilization of the provided form of chitin (Svitil et al. 1997).

Among the multiple chitinases group A chitinases were shown to be expressed in the largest amounts and to be the most active enzyme toward insoluble chitin (Suzuki et al. 2002, Orikoshi et al. 2005). Thus, most studies on the diversity and distribution of bacterial chitinases in diverse environments have been focused on family 18 group A chitinases (Cottrell et al. 2000, Ramaiah et al. 2000, Hobel et al. 2005, Lindsay et al. 2010). The dominance of group A chitinase (*chiA*) genes in aquatic environments was recently demonstrated by Beier et al. (Beier et al. 2011). Compared to the number of studies on bacterial chitinases in marine and saline ecosystems (Svitil and Kirchman 1998, Cottrell and Kirchman 2000, Ramaiah et al. 2000, LeCleir et al. 2004, LeCleir et al. 2007, Beier et al. 2011) the number of studies on bacterial chitinases in freshwater ecosystems is low and mostly focused on environments with extreme characteristics (Hobel et al. 2005, Xiao et al. 2005, LeCleir et al. 2007). Bacterial chitinases present in freshwater ecosystems have been
reported to be related to Firmicutes, β- and γ-Proteobacteria, Bacteroidetes (Flavobacteria, Cytophaga), and Actinobacteria (Hobel et al. 2005, Xiao et al. 2005, Beier and Bertilsson 2011).

Based on 16S rRNA gene approaches, β-Proteobacteria and Actinobacteria were identified as predominant bacterial groups in freshwater lakes (Hiorns et al. 1997, Glöckner et al. 2000, Humbert et al. 2009, Oh et al. 2011). Actinobacteria have previously been described as efficient chitinolytic agents in soil habitats, in particular the genus Streptomyces (Metcalf et al. 2002, Bhattacharya et al. 2007, Hjort et al. 2010). However, a recent study, which reports the consumption of chitin hydrolysis products by Actinobacteria without being actively involved in the hydrolysis of chitin, did not find evidence for a role of this group as key players for the hydrolysis of chitin in freshwater lakes (Beier and Bertilsson 2011).

In a previous study, we investigated the chiA gene abundance and the chitin turnover in zooplankton, the water column and the sediments of oligotrophic Lake Brienz (LB) and eutrophic, partially anoxic Lake Zug (LZ) (Köllner et al. 2012). Chitinase activity was found in the sediments and associated with zooplankton. In the oligotrophic water column, a significantly higher abundance of chiA genes was detected compared to the eutrophic waters of LZ. For the sediments of both lakes, significant correlations between chiA gene abundance and chitin turnover were detected. Based on these findings and the literature we postulated that the sites of chitinolytic activity would harbor a high diversity of chitinolytic bacteria and would therefore yield diverse chitinase sequences. Our previous work also raised the question whether the potentially chitinolytic planktonic bacterial community (0.2 to 5 µm fraction) consist largely of microbes detaching from chitin-containing particles and would therefore show similarity to the composition of the zooplankton associated community. Alternatively these bacteria could be organisms with a planktonic lifestyle that may primarily profit from the uptake of chitin hydrolysis products as proposed for planktonic Actinobacteria (Beier and Bertilsson 2011). Based on our knowledge on the global distribution of freshwater taxa (Glöckner et al. 2000, Zwart et al. 2002), we further hypothesized that similar chitinolytic bacterial assemblages would be present in both lakes.

In order to test these hypotheses and to reveal the key players for the hydrolysis of chitin in the diverse habitats of freshwater lakes, we applied 454 pyrosequencing of 16S rRNA and chiA genes on selected samples, for which high
3.3 Methods

3.3.1 Sampling

The sampling procedure and the characteristics of the sampled lakes are described in detail elsewhere (Köllner et al. 2012). Briefly, LB is an oligotroph, fully oxic lake with a maximum depth of 259 m and was sampled in mid-May and mid-September 2009. The South Basin of eutrophic LZ is meromictic and anoxic below 130 m. It has a maximum depth of 200 m. LZ was sampled end of March and end of October 2009. For each lake, water was sampled from ten different depths at the deepest point of the basin. Sediment cores were recovered from the two sampling sites using a gravity corer. The cores were sliced at intervals of 1 centimeter and the homogenized subsamples were stored at -80°C until processing. Zooplankton and phytoplankton were sampled during the fall sampling campaigns.

For 454 pyrosequencing of chiA gene sequences we selected samples, for which high chiA gene abundance was detected via quantitative PCR previously (Köllner et al. 2012). For LZ, we selected the 5-m and the 10-m water layers sampled in March and October 2009, respectively. For LZ, the number of chiA genes was below the limit of detection below the surface waters (Köllner et al. 2012). In the LB water column, chiA maxima were measured in the 5-m water layer for both samplings, in May and September 2009. High chiA gene abundance was also observed in the hypolimnion of LB, where it peaked in the 150-m water layer sampled in May 2009 and the 200-m water layer sampled in September 2009. Zooplankton from the fall sampling campaigns and the surficial (0 to 1 cm) sediments of both lakes and both sampling campaigns were also included. The same samples were also subjected to pyrosequencing of 16S rRNA genes. In addition, the 80-m water sample as representative for the oxic hypolimnion of LZ and the 170-m water depth as representative for the anoxic waters of LZ, both sampled in fall, were also subjected to 454 pyrosequencing of 16S rRNA genes. The description of the samples subjected to 454 pyrosequencing and the corresponding abbreviations are listed in Table 6.
Rare bacterial community members dominate the functional trait of chitin hydrolysis in freshwater lakes

Table 6. List of samples subjected to 454 pyrosequencing targeting the V1-V2 hypervariable regions of the 16S rRNA gene and the chitinase family 18 group A gene (chiA)

<table>
<thead>
<tr>
<th>Sample id&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sample description</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBS 5 m</td>
<td>5 m water depth of LB in May 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LBS 150 m</td>
<td>150 m water depth of LB in May 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LBF 5 m</td>
<td>5 m water depth of LB in September 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LBF 200 m</td>
<td>200 m water depth of LB in September 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LZS 5 m</td>
<td>5 m water depth of LZ in March 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LZF 10 m</td>
<td>10 m water depth of LZ in October 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LZF 80 m</td>
<td>80 m water depth of LZ in October 2009</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>LZF 170 m</td>
<td>170 m water depth of LZ in October 2009</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>LBS Sed</td>
<td>Surficial (0 to 1 cm) sediments of LB in May 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LBF Sed</td>
<td>Surficial (0 to 1 cm) sediments of LB in September 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LZS Sed</td>
<td>Surficial (0 to 1 cm) sediments of LZ in March 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LZF Sed</td>
<td>Surficial (0 to 1 cm) sediments of LZ in October 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LZF Zoo</td>
<td>Zooplankton ≥ 95 µm of LZ in October 2009</td>
<td>16S rRNA, chiA</td>
</tr>
<tr>
<td>LBF Zoo</td>
<td>Zooplankton ≥ 95 µm of LB in September 2009</td>
<td>16S rRNA, chiA</td>
</tr>
</tbody>
</table>

<sup>a</sup> LB, Lake Brienz, LZ, Lake Zug. S, Spring sampling, F, Fall sampling

3.3.2. DNA extraction

Total bacterial DNA from zooplankton (≥ 95 µm), water, and sediment samples were extracted as described previously (Köllner et al. 2012).

3.3.3. 454 pyrosequencing

Titanium Fusion Primers were used to amplify the 16S rRNA gene hypervariable V1-V2 region (Escherichia coli positions 27-338) from 14 samples (Table 6): The forward sequencing primer (454A)B-338R (5’-CGTATCGCCTCCCTCGGCATCGA<sup>ACCGATCGTCATGCTGCCTCCCGTAGG</sup> AGT-3’) consisted of the Titanium primer A sequence and one of 14 unique eight-base barcode (bold, italics) followed by a linker (italics) and the template-specific sequence (underlined). The reverse primer (454B)-27F (5’-CTATCGCCTTGGCAGCCGGCTCAGT<sup>TCAGAGTTTGATCCTGGCCTCAG</sup>-3’) consisted of the Titanium primer B sequence, a linker (italics) and the template-specific sequence (underlined).

The 14 unique barcode sequences (one for each sample) were taken from (Hamady et al. 2008) and selected to maximize all way dissimilarity between
barcodes. Additionally, the primers were checked for secondary structures formation on http://eu.idtdna.com/Scitoools/Applications/unafold/.

The PCR reaction mix contained 1x PCR buffer (Promega, Madison, WI), 0.2 µM of each primer (Microsynth, Balgach, Switzerland), 0.2 mM of each dNTP (Promega), 3 mM MgCl₂, (Microsynth), 0.5 g l⁻¹ bovine serum albumin (Sigma-Aldrich Chemie GmbH, Switzerland), 1 U Taq polymerase (Promega), and nuclease – free water (Qiagen AG, Switzerland) in a final volume of 50 µl. The PCR cycling regime consisted of an initial denaturation step at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min and ending with an extension step at 72°C for 10 min.

For the specific amplification of chiA gene fragments of an expected size of 430 bp, Titanium Fusion Primers carrying chiA – specific primers (Xiao et al. 2005) were used: The forward primer (454B)B-chir (5’-CTATGCGCCTTGCCAGCCCGCTCAGACGATCGTTCCSGTCCAGCCGCGSCC RTA-3’) consisted of the Titanium primer B sequence, one unique barcode (bold, italics), a linker (italics) and the chir sequence (underlined). The same barcode sequences as for the amplification of the 16S rRNA gene fragments were used. The reverse primer (454A)-chif2 (5’-CGTATCGCCTCCCTCGCCATCAGCAGACGGCATCGACATCGATTGG-3’) consisted of the Titanium primer A sequence, a linker (italics) and the template-specific sequence (underlined). Each chiA PCR reaction contained 1 x PCR buffer (Promega), 0.2 µM of each primer (Microsynth), 0.2 mM of each dNTP (Promega), 1.5 mM MgCl₂, (Microsynth), 1 g l⁻¹ bovine serum albumin (Sigma), 2 U Taq polymerase (Promega), 2 (water samples) or 10 ng (sediment and zooplankton samples) template, and nuclease – free water (Qiagen) in a final volume of 50 µL. The chiA PCR cycling regime consisted of an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 50 s, annealing at 65°C for 60 s, and extension at 72°C for 90 s and ending with an extension step at 72°C for 10 min.

Each sample was subjected to amplification of 16S rRNA and chiA gene fragments in triplicate. The PCR products were separated on a 2% agarose gel and the specific DNA fragments excised. The triplicate gel slices were pooled and cleaned using the Wizard SV gel and PCR Clean-up system (Promega) according to the manufacturer’s instructions. Cleaned products were quantified on a Nanodrop ND-1000 spectrophotometer (NanoDrop products (NanoDrop Technologies (Thermo
Fisher Scientific), Wilmington, DE). The 14 16S rRNA gene subsamples, each containing 200 ng of DNA, and the 12 \( \text{chiA} \) subsamples, each containing 120 ng of DNA, were combined, giving two amplicon pools. The amplicon mixes were further cleaned by gel purification and subsequently sequenced by Microsynth using Roche’s 454 Titanium sequencing platform. The 16S rRNA amplicon pool was sequenced on 3/16 run (expected number of reads: 72’000) and the \( \text{chiA} \) amplicon pool was sequenced on 1/16 run (expected number of reads: 24’000).

3.3.4. Data pre-processing and analysis of 16S rRNA gene sequences

76’278 16S rRNA gene sequences - the median sequence length was 356 bases - were trimmed using mothur 1.21.1 (Schloss et al. 2009): The sequences were screened for the barcode and the 338R primer sequence (forward primer). Sequences carrying homopolymers of more than 8 bases in length or ambiguous bases or low quality sequences with an average quality score below 25 were removed. 68’724 sequences passed this quality screening, i.e., ~90% of the original dataset, and 48’155 sequences were unique. The unique sequences were aligned against a SILVA-based bacterial reference alignment provided by www.mothur.org. Sequences were screened for correct position (end of the sequence at aligned position 6333) and maximum overlap, and columns containing only gaps were removed. Subsequently the alignment was minimized to the overlapping sequence space. Preclustering was used to reduce sequence noise (Huse et al. 2010). Next, sequences containing chimeras were scrapped. After phylogenetic classification using the mothur-formatted RDP taxonomy training set (http://www.mothur.org/wiki/RDP_reference_files), sequences assigned to \( \text{Archaea} \) (70 sequences) and sequences originating from chloroplasts (2’588 sequences) were excluded from further analysis. With this final pre-processing step the dataset still contained ~70% of the original dataset, i.e., 54’854 sequences. After computation of the distance matrix, the 16S rRNA sequences were clustered into operational taxonomic units (OTUs) using the average neighbor algorithm and sequence similarity cutoff of 97%, which was used as a proxy for species boundaries (Stackebrandt and Goebel 1994). Based on the 97% similarity OTU definitions, rarefaction curves and various diversity and richness metrics were calculated. The percentage of coverage was calculated by Good’s method with the formula \[ [1 – (n/N)] \times 100 \], where \( n \) is the number of OTUs in a sample represented by one sequence (single-read OTUs) and \( N \) is the total number of sequences in that sample (Good
3.3 Methods

For similarity analysis of bacterial communities between samples, dendrograms were constructed based on 16S rRNA gene OTUs. Bray-Curtis (Bray and Curtis 1957) and Yue & Clayton theta (Yue and Clayton 2005) similarity coefficients were applied.

3.3.5. Data pre-processing and analysis of chiA sequences

21'890 chiA sequences – the median sequence length was 432 bases - were trimmed using mothur. The sequences were screened for presence of forward and reverse primer motifs and sequences without match to both were removed. Any sequences containing homopolymers of more than 8 bases in length or ambiguous bases or low quality sequences that have an average quality score below 25 were likewise excluded from further analyses. 11'062 putative chiA sequences passed this quality screening. The sequences were translated in all three forward reading frames. The amino acid sequences were subjected to a BLASTP search (Altschul et al. 1997) against the protein database refseq_protein provided by http://blast.ncbi.nlm.nih.gov/ using the BLOSUM62 matrix and an E-value threshold of 1e-7. Only continuous amino acid sequences (no stop codons), which showed an alignment length of at least 100 amino acids and a percentage of identity of at least 45% with the reference sequences, were kept in the dataset. In a second step the 9'716 amino acid sequences which met these quality criteria, were blasted against a reference protein database containing 88 bacterial chitinase sequences spanning a broad range of taxa, applying the same screening criteria as above. Finally, 9'700 sequences showed alignments to bacterial chitinases in the refseq-protein database and exceeded the screening thresholds. The corresponding nucleotide sequences were selected from the original chiA pyrosequencing dataset and the unique sequences (4'076) filtered using mothur. The nucleotide sequences were aligned and used as seed alignment for the amino acid sequences. The alignments were created with the program HMMer v2.3 (Eddy 1998). Distance matrices were generated in Phylip format using ARB (Ludwig et al. 2004). OTU-based analyses were performed analogous to the analysis of the 16S rRNA gene sequences described above. As there exist contradicting reports regarding the correlation between the 16S rRNA phylogeny and the phylogeny of bacterial chitinases (Cottrell et al. 2000, Ramaiah et al. 2000, Xiao et al. 2005), the results were evaluated for different sequence similarity levels of 95%, 90%, and 80%. A neighbor-joining tree with the unique chitinase sequences (3’062) and 88 reference sequences
was constructed using ARB (Ludwig et al. 2004). Reference sequences were selected from GenBank to represent chitinases commonly described in the literature and according to BLASTP hits obtained for chitinases found in this study. Closely related sequences were grouped and the grouped sequences compared to the OTUs formed at the different sequence similarity levels. The majority of sequences that clustered in distinct branches in the ARB tree were also assigned to a specific OTU at each of the different sequence similarity levels used for OTU definition. For reasons of simplicity, results of chitinase sequence analysis are only shown for OTUs based on a sequence similarity level of 90%.

De novo phylogenetic trees, which contained sequences of abundant chitinase OTUs and a subset of reference sequences, were constructed using the program Mega 5.05. Abundant OTUs were defined as OTUs, which comprised > 3% of the sequences relative to the total chitinase sequence pool obtained for the respective sample. The sequences were aligned by ClustalW and phylogenetic trees were constructed based on the neighbor-joining, the maximum-parsimony, and the maximum likelihood method.

3.4. Results

3.4.1. Bacterial community structure

In total, 76’278 pyrosequencing reads of the V1-V2 region of the 16S rRNA gene were obtained from the different lakes and lake habitats (water, sediment, and zooplankton). Of this initial sequence pool, 54’854 high quality bacterial sequences were retained for analysis and assigned to bacterial phyla. For the water samples of LB and the oxic water samples of LZ, the dominant bacterial group was Actinobacteria (34% to 66% of all sequences) (Fig. 13). The second most abundant group was β-Proteobacteria (14% to 27%). Other abundant sequences belonged to the groups Bacteroidetes (up to 14%), α-Proteobacteria (up to 12%), and Cyanobacteria (up to 6%). γ-Proteobacteria became more abundant in the hypolimnion samples of LB (up to 8%) and LZ and in the anoxic water sample (LZF 170 m, 10%). The most abundant bacterial group for LZF 170 m was the β-Proteobacteria (38%), of which 67% were Burkholderiales, followed by Actinobacteria (27%) and Bacteroidetes (12%). Burkholderiales was the most abundant order within the β-Proteobacteria for all lake habitats.
In the sediment samples of LB, the dominant bacterial clade was the \(\beta\)-Proteobacteria (25% for both LBS and LBF), followed by the \(\alpha\)-Proteobacteria (13% (LBS) and 16% (LBF)). Other abundant bacterial groups were \(\gamma\)-Proteobacteria, Acidobacteria, Bacteroidetes, and \(\delta\)-Proteobacteria. In the sediment samples of LZ, the dominant bacterial clades were the \(\delta\)-Proteobacteria (22% (LZS) and 25% (LZF)), the Bacteroidetes (20% for both LZS and LZF) and the Firmicutes (8% (LZS) and 14% (LZF)).

Bacterial sequences obtained for the zooplankton sample of LB were dominated by Bacteroidetes (47%). In the zooplankton sample from LZ one quarter of the bacterial sequences belonged to Cyanobacteria and the other sequences were equally distributed over Alpha- (18%), Betaproteobacteria (12%), Gamma- (15%), \(\delta\)-Proteobacteria (10%), and Bacteroidetes (12%).

In contrast to the water samples, Actinobacteria were underrepresented in the sediments. They comprised only 3 to 5% of the obtained 16S rRNA gene sequences. Only two reads, one in LBS Sed and one in LZF Sed, were assigned to the genus Streptomyces, which is assigned a central role as chitin degrader in soil habitats and was also detected in Antarctic lake sediments (Xiao et al. 2005, Bhattacharya et al. 2007, Hjort et al. 2010, Nazari et al. 2011). However, within the Bacteroidetes high numbers of Flavobacteria sequences were detected, in particular for the zooplankton of LB, for which 41% of the 16S rRNA gene sequences were of the family Flavobacteriaceae. In all other samples, Flavobacteria represented from 1 to 7% (LZF Zoo) of the 16S rRNA gene sequences. Cytophaga sequences only comprised 1 to 4% of the 16S rRNA gene pool obtained for the diverse lake habitats. Flavobacteria together with Cytophaga are thought to play a crucial role in the hydrolysis of chitin in aquatic environments (Cottrell and Kirchman 2000, Beier and Bertilsson 2011). The most abundant taxonomic family within the Bacteroidetes in the hypolimnion and the surficial sediments of LB, for which high chiA gene abundances were detected in a previous study (Köllner et al. 2012), was the Chitinophagaceae.

For the well-known chitinolytic families Vibrionaceae (Svitil et al. 1997) and Enterobacteriaceae (Suzuki et al. 2002) of the class \(\gamma\)-Proteobacteria, only two and three reads, respectively, were detected. Aeromonadaceae (Lan et al. 2006) was only associated with LBF Zoo (3%) and Alteromonadaceae (Tsujibo et al. 1992) only with the sediments (~1% of the 16S rRNA gene sequence pool). In the present study most
of the $\gamma$-Proteobacteria sequences were obtained for the Xanthomonadaceae. Chitinolytic species belonging to Xanthomonadaceae have been described for lake sediments and soils (Metcalfe et al. 2002, Xiao et al. 2005, Hjort et al. 2010). Other chitinolytic bacteria described previously for freshwater belong to the phylum Firmicutes, e.g., Bacillus spp. (Hobel et al. 2005, Brzezinska and Donderski 2006). Sequences assigned to Firmicutes presented 3 to 14% of the total 16S rRNA gene sequence pool in the surficial sediments of both lakes and in zooplankton of LBF (3%), but were less present in the water samples. The most abundant class within the Firmicutes was however not Bacilli but Clostridia, which were prominent in the sediments (up to 12%). Chitinolytic Clostridia are known, although not commonly described for aquatic ecosystems (Timmis et al. 1974, Morimoto et al. 1999).

3.4.2. **OTU-based diversity analyses of 16S rRNA gene and chitinase sequences**

Distance matrices were generated for the 16S rRNA gene and the chitinase protein sequences using mothur and ARB (Phylip-formatted), respectively, and used to group 16S rRNA gene and chitinase sequences into OTUs. At a sequence similarity level of 97% (species level, (Stackebrandt and Goebel 1994)), 8’573 16S rRNA OTUs
were present in the complete dataset. 6'153 OTUs, i.e., 72%, were single-read OTUs. 6'934 OTUs, i.e., 81%, were unique to a single sample. The highest number of unique OTUs (1’597 OTUs) was found in LBF Sed.

The affiliation of chitinase sequences to OTUs at the sequence similarity levels of 95%, 90%, and 80% was compared to phylogeny observed in a neighbor-joining tree constructed with ARB from an alignment of 3’062 unique chitinase sequences. The majority of sequences that clustered in distinct branches in the ARB tree were also assigned to a specific OTU at each of the different sequence similarity levels used for OTU definition. At a sequence similarity level of 90%, 273 chitinase OTUs were detected. 163 OTUs, i.e. 60%, were single-read OTUs. 198 OTUs, i.e., 73%, were unique to a sample. As for the 16S rRNA gene sequences, by far the highest number of unique OTUs (71 OTUs) was found in LBF Sed.

For the 16S rRNA gene sequences, Good’s coverage was > 90% for the water samples and the zooplankton sample of LB. For the zooplankton sample of LZ, the coverage was 80%.

For the chitinase sequences, Good’s coverage was > 95% for the water samples and the zooplankton sample of both lakes although a mere 49 chitinase sequences was obtained for the zooplankton sample of LB.

For both the 16S rRNA gene and the chitinase sequences, the lowest Good’s coverage values were obtained for the sediment samples, especially for LB, which were < 60% for the 16S rRNA gene and < 87% for the chitinase sequences. Thus, the interpretation of the 16S rRNA gene and chitinase sequences for the sediments and the chitinase sequences for the zooplankton sample of LB was done with caution as the number of obtained sequences was too low to fully represent the diversity in the sampled population. Rarefaction curves for the 16S rRNA gene sequences and for the chitinase sequences are shown in Fig. 14.
Rare bacterial community members dominate the functional trait of chitin hydrolysis in freshwater lakes.

Figure 14. Rarefaction curves for the 16S rRNA gene sequences at a sequence similarity level of 97% (A) and for the chitinase sequences at a sequence similarity level of 90% (B) obtained for the water, sediment, and zooplankton samples of Lake Brienz (LB) and Lake Zug (LZ). Water samples were plotted as light grey lines, LB Sed as thin dashed lines, LZ Sed as thick dashed lines, LB FBF as thin black lines, and LB FZoo as thick black lines. Low number of chitinase sequences were obtained from sample LBF Sed, and LBF Zoo (49), and the corresponding rarefaction curve is therefore not visible on the scale used. For abbreviations and description of the samples see Table 6.
For both lakes, the diversity and richness of 16S rRNA genes and chitinase sequences in the surficial sediments far exceeded the water and zooplankton samples (Fig. 15). Comparing lake sediments, oligotrophic LB surpassed eutrophic LZ, in particular when comparing the diversity and richness of the chitinase sequences.
3.4.3. Similarity of 16S rRNA gene and chitinase OTUs between diverse lake habitats

In order to compare the similarity of bacterial communities and chitinases between diverse lake habitats, cluster analyses were performed based on chitinase and 16S rRNA gene OTU abundance data. The Bray-Curtis and the Yue & Clayton theta similarity coefficients featured essentially identical topologies. The dendrograms based on the Bray-Curtis similarity coefficient are shown in Fig. 16. Based on 16S rRNA gene OTU abundance data, the hypolimnion samples from LB in spring and fall were most similar and grouped with the oxic hypolimnion sample of LZ (Fig. 16A).

![Dendrogram](image)

Fig. 16. Dendrogram showing the similarity of 16S rRNA gene (A) and of chitinase (B) sequences between water, sediment, and zooplankton samples of Lake Brienz (LB) and Lake Zug (LZ) using shared OTUs for a sequence similarity level of 97% and 90%, respectively.
The anoxic communities of the 170 m sample of LZ were distinct from the rest of the water samples. For both lakes, the bacterial communities associated with the zooplankton and the sediment samples appeared to be distinct from the water samples, whereas the sediments of the same lake but from different seasons were grouped. In comparison, based on chitinase data, the sediment of LZ sampled in spring shared more OTUs with the spring surface water than with the sediment sampled in fall (Fig. 16B). For LZ, the most similar samples were the zooplankton and the surface water sampled in fall.

### 3.4.4. Chitinases detected in diverse lake habitats

While the total diversity across all samples was considerable, only a few OTUs dominated the chitinase libraries for each individual sample. Fig. 17 shows the distribution of abundant chitinase OTUs. Abundant OTUs were defined as OTUs, which comprised > 3% of the sequences relative to the total chitinase sequence pool obtained for the respective sample. 17 OTUs, which together accounted for 93% of the obtained chitinase sequences, achieved dominance according to this definition. Just two OTUs, i.e., OTU1, which was found predominant for the water samples of LB, and OTU2, which was found predominant in all habitats of LZ, together made up 86% of all sequences. However, the different library sizes and the varying coverage of the libraries are not considered in this perspective.

In all the LB water samples, 98% to 99% of the sequences were allocated to OTU1. Sequences of OTU1 could be assigned to a chitinase A sequence of the bacterial strain *Stenotrophomonas maltophilia* K279a (Fig. 18) sharing 87% to 100% sequence identity according to BLASTP. OTU1 also made up 12% of the chitinase sequences in the LZS 5-m water sample, but was not detected in any other LZ sample nor in the LB sediment and zooplankton samples. 16S rRNA gene sequences assigned to *Stenotrophomonas* were only detected in LB samples, except for one and two reads in LZS 5-m water and LZS sediment, respectively. The highest number of *Stenotrophomonas* sequences was obtained for the 5-m water sample of LBF (3% of the 16S RNA gene sequences obtained).
Rare bacterial community members dominate the functional trait of chitin hydrolysis in freshwater lakes

Fig. 17. Proportions of abundant chitinase OTUs for a sequence similarity level of 90% of water, sediment, and zooplankton samples of Lake Brienz (LB) and Lake Zug (LZ). Abundant OTUs were defined as OTUs, which comprised > 3% of the sequences relative to the total chitinase sequence pool obtained for the respective sample. For abbreviations and description of the analyzed samples see Table 6.

80% to 99% of the sequences in the surface water samples and the zooplankton sample of LZ were allocated to OTU2. OTU2 was the most abundant OTU also for the surficial sediments sampled in spring and fall, where it made up 65% and 41% of the sequences, respectively. Sequences of OTU2 were found to be most similar to *Bacilli* chitinases, but with less than 50% sequence identity according to BLASTP search against the protein database refseq_protein provided by http://blast.ncbi.nlm.nih.gov/. Thus, this OTU likely represents a novel bacterial chitinase lineage. For the zooplankton sample of LB, a mere 49 chitinase sequences were obtained. 45 were assigned to one unique OTU, i.e., OTU3, and the allocated sequences showed up to 79% similarity with actinobacterial chitinases according to BLASTP.

De novo phylogenetic trees were constructed with sequences representing each of the 17 abundant chitinase OTUs, related reference sequences according to BLASTP hits and reference chitinases commonly described for diverse environments.
Neighbor-joining, maximum-likelihood and maximum-parsimony algorithms resulted in essentially identical topologies. The neighbor-joining tree is shown in Fig. 18. The chitinase sequences were placed into three major clusters which were also observed in the ARB tree with the 3’062 unique chitinase sequences. In cluster I mainly chitinases detected for the water and sediment samples of LB, including OTU1, were grouped with known chitinases of the Xanthomonadaceae, specifically Stenotrophomonas. Cluster II contained OTU2, the predominant chitinase in the zooplankton, water, and sediment samples of LZ. In cluster III chitinases detected for the sediments of both lakes were grouped with known chitinases from Actinobacteria. Analyzing the affiliation of chitinases sequences that were present with more than 5 sequences per OTU and sample, instead of a detected proportion of > 3% per sample, additional chitinases affiliated with chitinases of Janthinobacterium lividum and of Micromonosporaceae were detected (Fig. 18). Less than 1% of the 16S rRNA gene samples obtained for a sample were assigned to the genus Janthinobacterium (maximum of 16 reads for LBS Sed). However, within the theoxic water samples the proportion of Burkholderiales was highest for the 5-m water samples of LBS (23%) and LZS (19%) (Fig. 18).
Rare bacterial community members dominate the functional trait of chitin hydrolysis in freshwater lakes

Fig. 18. Neighbor-Joining tree showing the phylogenetic affiliation of bacterial family 18 group A chitinases obtained from the different lake habitats studied here (bold). For abbreviations and description of the samples see Table 6. The number of sequences assigned to an OTU at a sequence similarity level of 90% is given in parentheses. Reference sequences were selected from GenBank to represent commonly described chitinases and according to BLASTP hits obtained for abundant chitinases found in this study. The GenBank accession number is given next to the source organism. Bootstrap values above 40 from 500 replicates are given at branch nodes. Cluster I contains abundant chitinase OTUs for the water and sediment samples of LB and known chitinases of Xanthomonadaceae, cluster II contains the most abundant chitinase OTU2 predominant in all samples of LZ. Cluster III contains chitinases detected for the sediments of both lakes and for the zooplankton of LB and known actinobacterial chitinases.
3.5. Discussion

In agreement with previous studies of diverse freshwater ecosystems and the suggested global distribution of freshwater bacterial clades (Glöckner et al. 2000, Zwart et al. 2002, Humbert et al. 2009, Vaz-Moreira et al. 2011), the dominant bacteria in the two contrasting lakes were *Actinobacteria*, *β-Proteobacteria* and *Bacteroidetes*. The bacterial communities sampled from the same water layer, i.e., epilimnion versus hypolimnion, but in different seasons were similar, with the anoxic water sample of LZ harbouring a unique bacterial community (Fig. 13 and Fig. 16). Water-layer-specific bacterioplankton communities were also observed for ARISA profiles of 16S rRNA gene fragments (Köllner et al., submitted). The spatial habitat heterogeneity – light-, temperature-, and oxygen- gradients, availability of organic matter and nutrients – was the main determinant shaping the bacterial communities in the lake water.

In contrast to the shared pattern of bacterial phyla in the water samples of the two studied lakes, the potential for chitin hydrolysis seemed to be mainly restricted to a single, but different, group of chitinases specific for each lake. The predominant group of chitinases in the waters of LB (OTU1) showed high similarity and for some sequences even identity to a chitinase A sequenced from *Stenotrophomonas maltophilia* K279a. *Stenotrophomonas maltophilia* strains were found to be prominent colonizers of chitin residues in soil, excreting a high level of chitinase (Krsek and Wellington 2001).

Another less abundant group of chitinases (< 3%) in the surface waters of both lakes could be identified as chitinases of *Janthinobacterium lividum* of the order *Burkholderiales* (Fig. 18). *Burkholderiales* was the most abundant order within the *β-Proteobacteria* for all lake habitats. In enrichment experiments on zooplankton chitin in surface water of LB and LZ, the enriched bacterial species were also assigned to families of the *Burkholderiales*, i.e., *Comamonadaceae* and *Oxalobacteraceae*, to the latter *Janthinobacterium lividum* belongs to (Wunderlin 2009). The detected chitinases appeared to originate from rare species in the bacterial communities of the diverse lake habitats, only up to 1 and 3% of the 16S rRNA sequences obtained were assigned to the genera *Janthinobacterium* and *Stenotrophomonas*, respectively. However, chitinase sequences assigned to *Janthinobacterium lividum* and
**Stenotrophomonas maltophilia** were also detected in Antarctic lake sediments (Xiao et al. 2005) and freshwater hot springs (Hobel et al. 2005).

In contrast to LB, the predominant group of bacterial chitinases in the water samples of LZ was also predominant in the zooplankton sample and the surficial sediments, and is thus more likely to represent a key player for chitin degradation in this lake. This group of chitinases could not be assigned to known bacterial chitinases. The sequences branched closest to a chitinase found in a species of the phylum *Bacteroidetes* (Fig. 18), but the similarity was low (36%).

In the sediment of LB sampled in fall, the majority of the chitinase sequences (51%) were allocated to actinobacterial chitinases (Fig. 18). Actinobacterial chitinases were also found in the surface water sample of LB in fall, however, they constituted a minor fraction (< 3%) compared to the total number of chitinase sequences detected for this sample and were not detected in the other water samples. The lack of actinobacterial chitinases in the lake water samples was contrary to our expectations. *Actinobacteria* are known as important chitinolytic agents especially for soil habitats (Metcalfe et al. 2002, Bhattacharya et al. 2007) and chitinolytic *Actinobacteria* were also isolated from lake sediments (Xiao et al. 2005). Since *Actinobacteria* were, in agreement with previous findings (Glöckner et al. 2000, Humbert et al. 2009), abundant in the studied lakes according to our 16SrRNA gene based analysis, we expected to detect actinobacterial chitinases in the water samples of both lakes, but this was not the case. In accordance with the observed lack of actinobacterial chitinases in the water samples, a recent study provided evidence of commensal planktonic *Actinobacteria* in lakes lacking the ability to hydrolyze chitin but profiting from the chitin hydrolysis products supplied by other bacterial groups (Beier and Bertilsson 2011). Beier et al. suggested *Flavobacteria* as significant chitinolytic agents in freshwater lakes as they were observed in dense clusters on chitin particles (Beier and Bertilsson 2011).

Whereas the predominant chitinases in the water columns were distinct between LB and LZ, some chitinase OTUs detected for the sediments were assigned to similar actinobacterial chitinases (Fig. 18), despite of the contrasting sediment properties. Apart from the contrasting trophic levels of the water columns the 0- to 1-cm sediment layer of LB is still oxic (Müller et al. 2007), whereas the LZ sediments are anoxic. However, for the sediments of both lakes, chitinase activity was detected in a previous study, in contrast to the water samples, for which the chitinase activity
was below the limit of detection (Köllner et al. 2012). In the sediments, elevated concentrations of chitin of diverse origin - detrital material originating from algae (diatoms) and arthropods (insects and crustaceans), crustacean molts and shed insect exoskeletons – are expected to be deposited. In general, sediments (as well as soils) harbor a far larger microbial diversity compared to aquatic habitats (Torsvik et al. 2002). Accordingly, it is not surprising that the highest diversity of bacterial chitinases was detected in the sediments of the studied lakes.

However, for the diverse lake habitats studied here, the ability to hydrolyze chitin seemed to be dominated by a restricted number of bacterial species. Moreover, the presented evidence suggests that chitinolytic agents were comparatively rare members of the total bacterial communities detected in the diverse lake habitats, at least for oligotrophic LB as we could not assign the predominant chitinases of LZ to a bacterial species. Pronounced dominance of single chitinolytic phylotypes was observed both in zooplankton and water samples. This is in agreement with results from a recent publication that showed that in microcosm experiments with lake water, the absence of a certain species was correlated with a loss of the function of chitin and cellulose degradation (Peter et al. 2011). This would indicate that planktonic freshwater bacterial communities themselves harbor a limited diversity of chitinolytic bacteria. Probably, a much higher number of organisms are able to metabolize the hydrolysis products released to the water column by chitinases acting on chitinous particles settling through the water column. It is intriguing in this respect that the dominant group of water column chitinases in LZ also dominated the zooplankton-associated library, while this was not the case in LB. In LB the water-dominant OTU1 was abundant neither in sediment nor in the zooplankton sample, the main sites of chitinolytic activity (Köllner et al. 2012). The importance of bacterial attachment to and from particles, e.g., zooplankton, has been demonstrated, so one would expect a certain overlap in species composition (Grossart et al. 2010). For LZ, up to 14 and 25% of the chitinase OTUs detected in the sediment (spring) and the zooplankton sample, respectively, were also detected in the surface waters (Fig. 16B). In contrast however, in LB the dominant Stenotrophomonas-like chitinases which were not detected in the zooplankton sample must belong to organisms with a different ecological strategy. It is possible that the host organisms are profiting from chitinase hydrolysis products released elsewhere rather than being directly involved in chitin degradation, similar to what has been reported for Actinobacteria (Beier and
Rare bacterial community members dominate the functional trait of chitin hydrolysis in freshwater lakes (Bertilsson 2011). Further research is required to determine the cause of these contrasting results.

Of course, as with all PCR-based analyses, preferential amplification of specific DNA fragments could have influenced the low evenness and diversity of chitinase sequences observed in this study. This is a caveat inherent also to 454 pyrosequencing (Vaz-Moreira et al. 2011) and results should not be interpreted quantitatively without extreme caution. However, the observed differences between the studied environments are so pronounced that we do not consider it likely that they are based on methodological artifacts. Another limitation is the relatively short read length of the current 454 pyrosequencing methodology (300 to 500 bp), which implicates a lower level of phylogenetic resolution compared to clone libraries of larger gene fragments, e.g. from near full-length rRNA gene amplification. On the other hand, even large clone libraries (100s of clones) generally fail to recover the total diversity of the targeted microbial communities (Pearce 2008, Jones et al. 2009) and are furthermore very time-consuming and laborious. The merits of 454 pyrosequencing lie in the detection of a high number of sequences and rare sequences with a comparatively small expense of time and labor. However, the bioinformatics required for analysis, specifically of functional gene sequences, is currently not trivial.

Sequences of bacterial chitinases are highly diverse probably due to lateral gene transfer between organisms (Hunt et al. 2008). Thus, it is impossible to amplify all the bacterial chitinases with a single pair of PCR primers. Previous studies on bacterial chitinases targeted subsets of bacterial chitinases by using specific PCR primers or combined sets of different primer pairs (Cottrell et al. 2000, Williamson et al. 2000, Hobel et al. 2005). We applied a primer pair, which was reported to target chitinase family 18 group A (chiA) gene fragments from a broad range of chitinolytic bacteria, including species of Actinobacteria, β- and γ-Proteobacteria and the Cytophaga-Flavobacterium-Bacteroides group (Xiao et al. 2005), and the capacity to amplify a broad range of chitinase genes was confirmed by our findings. However, we cannot exclude that the applied primer pair failed at or negatively biased the amplification of significant groups of bacterial chitinases, e.g., flavobacterial chitinases.

To summarize, whereas the two contrasting lakes shared the predominant bacterial classes, the diversity and distribution of the detected bacterial chitinases were found to be distinct between lakes and habitats. This suggests that the
chitinolytic bacteria are a functional group that is strongly shaped by the environmental parameters and ecological conditions of its habitat. Whereas the detected chitinases in the diverse lake habitats of LB could be assigned to chitinases of *Stenotrophomonas maltophilia*, *Janthinobacterium lividum* and *Actinobacteria*, the predominant group of chitinases in LZ could not be assigned to known bacterial chitinases. Future work has to isolate the chitinolytic bacteria or obtain genomic information by other means. In conclusion, as a restricted number of bacterial chitinases, which were assigned to rare members of the bacterial communities in the diverse lake habitats, was detected. We propose that their presence is essential for the functional trait of chitin hydrolysis in freshwater lakes.
4. IMPACT OF AMINO COMPOUNDS AND ORGANIC MATTER DEGRADATION STATE ON THE VERTICAL STRUCTURE OF LACUSTRINE BACTERIA

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submitted to *Aquatic Microbial Ecology*

Contributions of the authors:
The manuscript was written by Krista Köllner. Krista Köllner did the microbial and the statistical analyses and the interpretation of the data. Dörte Carstens measured the concentrations of amino compounds, analyzed the CI (based on chlorophyll) and the DI (based on protein amino acids) and contributed to the interpretation of the data. Carsten Schubert and Josef Zeyer significantly contributed to the interpretation of the data and corrected the manuscript. Helmut Bürgmann corrected the manuscript and significantly contributed to the conception and the interpretation of the presented study.
4.1. Abstract

In order to determine how concentration, composition, and degradation state of particulate organic matter (POM) influence lacustrine bacteria, we analyzed the changes in bacterial abundance and community structure along the water columns of two contrasting deep lakes, both located in Switzerland. Lake Brienz is oligotrophic and fully oxic while Lake Zug is eutrophic and partially anoxic. Automated ribosomal intergenic spacer analysis was conducted for free-living (> 0.2, < 5 µm) and particle-associated (> 5 µm) bacteria. Cluster analysis showed that the lakes comprised distinct bacterial communities (BCs). However, the BCs of both lakes were structured with depth. For the free-living BCs, redundancy analysis and forward selection of explanatory variables identified the Chlorin Index, an indicator for the degradation state of primary produced POM, and temperature as the main environmental determinants of the vertical community shifts. In contrast, the particle-associated BCs appeared less influenced by the POM degradation state and more directly affected by seasonal dynamics. For both bacterial size fractions, other degradation indices such as the ratios between particulate amino acids and particulate amino sugars performed less well as predictors for the BC structure. For both lakes, the bacterial cell number correlated significantly with the concentrations of particulate amino compounds and the Chlorin Index. The present study shows that not only the amount of organic matter, but also its degradation state shape the abundance and composition of lake bacterioplankton.

4.2. Introduction

One of the big challenges in aquatic microbial ecology is to derive the most important environmental factors that structure the bacterial community composition (BCC) and to relate the BCC dynamics to specific ecosystem functions. This is a prerequisite for creating predictive aquatic ecosystem models. Culture-independent molecular methods have facilitated the characterization of bacterial communities (BCs) in lakes over the last decades (Hiorns et al. 1997, Casamayor et al. 2000, Lindström 2000, Van Der Gucht et al. 2001, Zwart et al. 2002, Yannarell and Triplett 2004, Newton et al. 2011). By now, several studies are available that have determined environmental factors that constrain the BCC in these freshwater systems. For
instance, the availability of nutrients and organic carbon (Yannarell and Triplett 2004) and the physicochemical characteristics of a lake, such as pH, temperature (Methé & Zehr 1999, Lindström et al. 2005, Rösel et al. 2012), and oxygen concentrations (Shade et al. 2008) have been reported to shape the BCC. Biotic factors such as bacterivorous grazing, phage dynamics, and phytoplankton succession have also been demonstrated to determine BCC (Jürgens et al. 1999, Šimek et al. 2001, Kent et al. 2004, Kent et al. 2006, Salcher et al. 2011, Zeng et al. 2012).

A crucial function of aquatic bacteria is the degradation of organic matter and the resulting recycling of nutrients and carbon (Azam et al. 1983, Sherr and Sherr 1991). 30-60% of the planktonic primary production is mineralized by heterotrophic bacteria (Biddanda et al. 1994, Del Giorgio et al. 1997). In aquatic environments, the organic matter is divided into dissolved organic matter (DOM) and particulate organic matter (POM). In lakes, the POM fraction is estimated to contribute approximately 10% of the total organic matter and mainly consists of polymeric, high molecular weight compounds (Siuda and Chróst 2002). Amino sugars (ASs) and amino acids (AAs) are building blocks of a number of these biopolymers. Among these biopolymers are proteins, polysaccharides exuded from phytoplankton (Giroldo et al. 2003) and chitin, which is synthesized by diverse aquatic organisms (crustaceans, fungi, diatoms).

ASs and AAs have been used to indicate the quality and origin of POM. For instance, the relative abundance of specific particulate ASs and AAs can indicate the degradation state of POM (Lee and Cronin 1984, Haake et al. 1992, Dauwe and Middelburg 1998). In a previous study on the lakes studied here (see below), the ratios between the particulate ASs glucosamine (GlcN) and galactosamine (GalN) showed a strong decline below the zone of primary production (Carstens et al. 2012). This finding was related to a shift from the AS signature of organisms of higher trophic levels in the euphotic zone towards the signal of heterotrophic microorganisms and progressing diagenesis. Relative to ASs, AAs are preferentially utilized and, thus, the ratios between AAs and ASs decrease during microbial decomposition (Dauwe and Middelburg 1998, Davis et al. 2009).

A different measure for the degradation state of primary produced pigment related POM is the Chlorin Index (CI) introduced by Schubert et al. (2005). The CI increases for degraded material and has proven to be a powerful tool to characterize

Organic particles were identified as hot spots of microbial activity. Particle-associated bacteria were shown to hydrolyze more organic matter than they take up and, thus, they are considered to provide significant growth substrates for free-living bacteria in the surrounding waters (Smith et al. 1992, Grossart and Simon 1998a). As specific bacterial guilds are specialized in the degradation of certain substrates (Peter et al. 2011), it would be expected that organic matter quality and composition have a strong impact on the structure of BCs. Shifts in freshwater BCC related to organic matter quality have been shown previously (Crump et al. 2003, Roiha et al. 2011).

Recently published studies on diverse temperate lakes revealed distinct dynamics of particle-associated (> 5 µm) and free-living (> 0.2, < 5 µm) BCs in relation to biotic and abiotic factors (Allgaier et al. 2007, Parveen et al. 2011, Rösel et al. 2012). However, at present our understanding of the interactions between organic matter and BCC during the sedimentation process in deeper lakes is very limited.

In the present study, we analyzed the vertical changes in bacterial assemblages by automated ribosomal intergenic spacer analysis (ARISA) in two contrasting deep lakes distinguishing themselves by their nutrient loads and redox conditions. The two lakes sampled in spring and fall 2009 are Lake Brienz (LB, oligotrophic, fully oxic) and Lake Zug (LZ, eutrophic, partially anoxic), both located in Switzerland. We separated the free-living and the particle-associated BCs via serial filtration through 5.0 and 0.2 µm pore size filters. Bacterial cell numbers were determined along the lake water column via flow cytometry. The BCC patterns were linked to bulk parameters such as total organic carbon (TOC), oxygen (O₂), pH, temperature (T), and to various parameters indicating POM degradation state and composition, i.e., the CI and the abundance and ratios of specific particulate amino compounds.

We hypothesize that shifts in the composition and degradation state of POM in a lake water column are accompanied by shifts in bacterial abundance and BCC. Further we hypothesize that distinct environmental parameters shape the BCs in an oligotrophic, fully oxic water column compared to a eutrophic, partially anoxic water column. Finally, different structure-function relationships are expected for particle-associated versus free-living BCs.
4.3 Methods

4.3.1 Sampling sites

The characteristics of the lakes are described in detail elsewhere (Köllner et al. 2012). Briefly, LB is an oligotroph, fully oxic lake with a maximum depth of 259 m. The South Basin of eutrophic LZ is meromictic and anoxic below 130 m. It has a maximum depth of 200 m. Results for basic chemical and physical parameters, with the exception of alkalinity, ion concentrations, and pH, were reported previously (Carstens et al. 2012, Köllner et al. 2012) and are shown in Fig. 6 and Table 9.

4.3.2 Sampling

The sampling procedure is described in detail elsewhere (Köllner et al. 2012). Briefly, LB was sampled in mid-May and mid-September 2009 and the South Basin of LZ was sampled end of March and end of October 2009. Based on T and O₂ profiles (Fig. 6), water from LB was sampled at depths of 5, 10, 20, 30, 40, 70, 100, 150, 200, and 240 m and from LZ at 5, 10, 15, 25, 60, 80, 100, 130, 170, and 190 m. For both lakes the samples were collected over the deepest point of the basin. For AA, AS, and CI analyses (described below) POM from the same depths was sampled onto two stacked precombusted glass fiber filters (nominal pore size 0.7 μm, 142 mm diameter; Whatman Inc., Florham Park, NJ) with in-situ pumps (McLane Research Laboratories Inc., Falmouth, MA) until filters were clogged.

4.3.3 Chemical analysis

Concentrations of TOC and total nitrogen (TN) were measured as described previously (Köllner et al. 2012). Total phosphorus (TP) concentrations were determined photometrically in unfiltered water samples with the molybdenum blue method of Vogler (1965). Alkalinity was analyzed by titration with 0.1 N HCl to pH 4.3 with an automatic titration system (716 DMS Titrino, Metrohm AG, Zofingen, Switzerland). For determination of chloride (Cl⁻), nitrate (NO₃⁻), and sulfate (SO₄²⁻) concentrations, aliquots were filtered through cellulose acetate filters with 0.45 μm pore size (Whatman). Analysis was performed using a Metrohm ion chromatograph (3.2 mM Na₂CO₃/1.0 mM NaHCO₃ buffered solution, 0.7 ml min⁻¹ flow rate) and a Metrohm Anion Metrosep A Supp 5 column. Concentrations of the base cations calcium (Ca²⁺), magnesium (Mg²⁺), and sodium (Na⁺) were also measured with a
Metrohm ion chromatograph (1.7 mM nitric acid/0.7 mM dipicolinic acid eluant, 0.9 ml min$^{-1}$ flow rate) equipped with a Metrohm Metrosep C4 column.

4.3.4. Amino acid analysis

The concentrations of 14 particulate protein AAs, alanine (Ala), glycine (Gly), threonine (Thr), serine (Ser), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), aspartic acid (Asp), methionine (Met), glutamic acid (Glu), phenylalanine (Phe), tyrosine (Tyr), lysine (Lys), and the two nonprotein AAs $\gamma$-aminobutyric acid ($\gamma$-aba) and ornithine (Orn) were measured. For this purpose, one quarter of each 0.7 µm filter was hydrolyzed with 6 mol l$^{-1}$ HCl for 20 hours at 110°C under N$_2$. Prior to hydrolysis, L-norleucine (Sigma) was added as internal standard. The samples were processed as described previously for the analysis of particulate D-AAs (Carstens et al. 2012). A standard mixture of AAs with known concentration (AA S-18 (Sigma), L-norleucine (Sigma), ornithine (Sigma), $\gamma$-aba (Sigma)) was also derivatized and injected on the GC for quantification.

4.3.5. Degradation index

The degradation index (DI) was calculated based on the first axis of a principle component analysis (PCA) of mole percentages of the detected particulate protein AAs according to Dauwe et al. (1999).

4.3.6. Amino sugar analysis

Concentrations of the particulate ASs, GlcN, mannosamine (ManN), GalN, and muramic acid (MurA) on 0.7 µm filters were analyzed according to the method of Zhang & Amelung (1996) and Guerrant & Moss (1984) with slight modifications as described previously (Carstens et al. 2012).

4.3.7. Chlorin Index

The CI was determined on aliquots of the 0.7 µm filters as described elsewhere (Schubert et al. 2005, Bechtel and Schubert 2009). Briefly, the filters were extracted with acetone 3 times and the fluorescence was measured at an excitation wavelength of 428 nm and an emission wavelength of 671 nm. The extracts were acidified with 100 µl of 25% HCl and the fluorescence was measured again. The fluorescence intensity of the acidified sample was divided by the fluorescence
intensity of the non-acidified original extract which is defined as the CI. The CI of fresh chlorophyll \( a \) is 0.2 and increases for degraded organic matter up to unity.

### 4.3.8. Bacterial cell counts

Bacterial cells were counted using flow cytometry as described previously (Carstens et al. 2012).

### 4.3.9. DNA extraction

To separate the free-living and the particle-associated BCs about five liters of water from each sampled depth were filtered through a 5.0 \( \mu \)m isopore membrane filter (Millipore, Billerica, MA) and a 0.2 \( \mu \)m polycarbonate filter (Whatman), each 142 mm in diameter, connected in series and processed for DNA extraction as described previously (Köllner et al. 2012).

The quality of DNA extracts was checked by agarose gel (1%) electrophoresis. Extracted DNA was quantified by fluorescence spectroscopy using the Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes) and a Synergy HT microplate reader (Bio-Tek Instruments Inc., Winooski, VT).

### 4.3.10. Amplification of ribosomal intergenic spacer fragments

The ARISA-PCR mixture (50 \( \mu \)l) contained 1 x PCR buffer (Promega, Madison, WI), 2.5 mmol l\(^{-1}\) \( \text{MgCl}_2 \) (Promega), 0.25 mmol l\(^{-1}\) of each dNTP (Promega), 0.25 g l\(^{-1}\) bovine serum albumin (Sigma), 10 ng of extracted DNA, 400 nmol l\(^{-1}\) of primers (Microsynth, Balgach, Switzerland) 1406f (5’-TGYACACACCGCCCGTTACG-3’) and 23r (5’-GGGTBCCCCATTACG-3’) targeting bacterial 16S rRNA genes (Lane 1991), and 1.25 U \( \text{Taq} \) polymerase (Promega). The forward primer was labeled at the 5’ end with fluorescent dye 6-FAM (Yannarell et al. 2003). ARISA PCR was performed on a Techne TC-512 thermocycler (Witec AG, Littau, Switzerland) as described previously (Yannarell et al. 2003). The size and quality of the PCR product was checked by agarose gel (2%) electrophoresis. Each PCR sample was subjected to capillary electrophoresis on an ABI Prism 3130xl genetic analyzer (Applied Biosystems, Foster City, CA) as follows: 1 \( \mu \)l of ARISA-PCR product was added to a master mix containing 0.5 \( \mu \)l of internal size standard LIZ 1200 (20–1200 bp) (GeneScan, Applied Biosystems) and 9 \( \mu \)l of deionized Hi-Di formamide (Applied Biosystems), denatured for 3 min at 95°C and placed on ice.
immediately for at least 5 min. The ARISA-PCR products were separated by capillary electrophoresis on 50 cm capillaries using the POP-7 polymer and the following run parameters: 15 kV (run voltage), 2.4 kV (injection voltage), 20 s (injection time), and 60°C (oven temperature). Electropherograms were analyzed using GeneMapper software v 4.0 (Applied Biosystems). The minimum peak height was 150 fluorescence units for the sample signal and 15 fluorescence units for the Liz standard signal. The capillary electrophoresis was performed with triplicate ARISA-PCR samples of the particle-associated BCs and quadruplicate ARISA-PCR samples of the free-living BCs.

4.3.11. Binning

ARISA fragments between 390 and 1250 bp in size were analyzed using published R (R Development Core Team 2009) binning scripts (Ramette 2009). Only peaks with relative fluorescence intensity values of > 0.09% and a consistent presence in two (particle-associated) to three (free-living) ARISA-PCR replicates were included in further analyses. In order to determine the best window size (WS) the automatic R binning script, available online (http://www.mpi-bremen.de/en/Software_2.html), was applied to replicates of at least one representative sample (highest number of ARISA fragments) for each sampling and both bacterial size fractions. The correlation between the replicates and the respective number of operational taxonomic units (OTUs) for a series of WS values (0.5, 1, 1.5, 2, 3, 4, and 5 bp) and a shift value (Sh) of 0.1 bp was calculated. As a compromise between high resolution and high similarity between sample replicates, a WS of 3 bp was used for the OTU binning algorithm for ARISA profiles from both the free-living and the particle-associated BCs.

4.3.12. Statistical analysis

All statistical analyses were performed using the statistical software R version 2.14.0 (R Development Core Team 2009) with packages VEGAN (Oksanen et al. 2011) and BIODIVERSITY R (Kindt and Coe 2005). For cluster analysis, average linkage of hierarchical cluster analysis was used with the distance among communities calculated as Bray-Curtis distances. Prior to principal component analysis (PCA) and redundancy analysis (RDA), the ARISA OTU data were Hellinger-transformed (Legendre and Gallagher 2001, Ramette 2007) and
environmental data were z-standardized to offset different units and scales. The environmental variables used for PCA and RDA and their corresponding abbreviations are given in Table 7. RDA is a multivariate multiple linear regression followed by a PCA of the response matrix of fitted values (Borcard 2011). For each RDA, the adjusted $r^2$ ($r^2_{adj}$) was calculated based on Ezekiel’s formula (Ezekiel 1930), which adjusts the $r^2$ value according to the number of parameters in the model as an increased number of variables inflates $r^2$ (Peres-Neto et al. 2006).

For variable reduction and in order to create an efficient model from the most significant explanatory variables, forward selection of constraints using the `forward.sel` function of the R package packfor (Dray et al. 2009) and VEGAN’S `ordistep` function were applied. With `forward.sel`, variables are included stepwise in the explanatory model, starting with the variable which explains most of the variance (partial $r^2$) of the response data (ARISA data) and has a significant contribution to the model ($p < 0.05$). The test of significance is a permutation test by partial RDA. The forward selection stops after the significant variable by which the $r^2_{adj}$ of the global model was exceeded has been added. The `ordistep` function was also used for forward variable selection. This algorithm alternates between stepwise addition and deletion of explanatory variables until the model is stable. Models were tested by permutation analysis (1000 iterations).

In order to compare ARISA OTUs of the free-living and the particle-associated BCs multiple response permutation procedure (MRPP) was computed using the R package VEGAN.

Environmental variables, which showed correlation to bacterial cell counts with a $r^2$ of $\geq 0.60$ and a $p$-value of $\leq 0.05$ for at least two sampling dates were included as explanatory variables for cell counts in multiple regression analysis using R.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total organic carbon</td>
<td>TOC</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>TN</td>
</tr>
<tr>
<td>Ratio between total organic carbon and total nitrogen</td>
<td>C/N</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>TP</td>
</tr>
<tr>
<td>Nitrate</td>
<td>NO$_3$</td>
</tr>
<tr>
<td>Sulfate</td>
<td>SO$_4^{2-}$</td>
</tr>
<tr>
<td>Calcium</td>
<td>Ca$^{2+}$</td>
</tr>
<tr>
<td>Sodium</td>
<td>Na$^+$</td>
</tr>
<tr>
<td>Chloride</td>
<td>Cl$^-$</td>
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<tr>
<td>Magnesium</td>
<td>Mg$^{2+}$</td>
</tr>
<tr>
<td>Alkalinity</td>
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</tr>
<tr>
<td>Temperature</td>
<td>T</td>
</tr>
<tr>
<td>Particulate glucosamine</td>
<td>GlcN</td>
</tr>
<tr>
<td>Particulate mannosamine</td>
<td>ManN</td>
</tr>
<tr>
<td>Particulate galactosamine</td>
<td>GalN</td>
</tr>
<tr>
<td>Particulate muramic acid</td>
<td>MurA</td>
</tr>
<tr>
<td>Particulate glutamic acid</td>
<td>Glu</td>
</tr>
<tr>
<td>Ratio between particulate glucosamine and particulate galactosamine</td>
<td>GlcN/GalN</td>
</tr>
<tr>
<td>Sum of particulate amino acid concentrations divided by the sum of particulate amino sugar concentrations</td>
<td>AAs/ASs</td>
</tr>
<tr>
<td>Chlorin Index</td>
<td>CI</td>
</tr>
</tbody>
</table>

*Parameters used for redundancy analysis (RDA).

*As strong correlation between the various AAs was observed (Fig. 22), Glu was used as representative of the 14 protein AAs measured. For abbreviations of other individual AAs evaluated in this study see chapter “Amino acid analysis” and http://www.ncbi.nlm.nih.gov/Class/MLACourse/Modules/MolBioReview/iupac_aa_abbreviations.html.
4.4 Results

4.4.1. Richness and community structure of free-living and particle-associated bacteria

Based on the number of detected ARISA OTUs, the richness was not significantly different between the two lakes, whether considering the free-living or the particle-associated BCs (t-test, $\alpha = 0.05$, $p = 0.80$ (free-living) and $p = 0.60$ (particle-associated)). When performing a pairwise comparison per sampling depth (separately for each sampling date and lake), the richness of the free-living BCs was significantly higher compared to the particle-associated BCs (pairwise t-test, $\alpha = 0.05$, $p < 0.0001$) with the exception of LZ in October (t-test, $\alpha = 0.05$, $p = 0.08$). The number of ARISA OTUs per profile ranged from 48 ± 1 to 75 ± 2 and from 21 ± 1 to 69 ± 1 for the free-living and the particle-associated BCs, respectively.

The cluster analysis of the ARISA profiles of free-living BCs grouped LB and LZ into separate clusters (Fig. 19). Within the LB cluster, the hypolimnion was distinct from the epilimnion. The LB hypolimnion cluster included the 70 to 240 m water depth of the spring sampling and the 40 to 240 m water depth of the fall sampling. This finding is in good agreement with the recorded conductivity-temperature-depth profiles (Fig. 6A,B): In spring, T was constantly 5°C below a water depth of 70 m while in fall T was constantly 6°C below a water depth of 40 m. The second largest cluster was formed by the oxic waters of LZ. The 25 m water depth sampled in October was distinct from the epilimnion and grouped with the water depths sampled along the oxycline of LZ (see Fig. 6C,D). The anoxic water layers of LZ formed an outgroup.

As observed for the free-living BCs, the particle-associated BCs of LB and LZ were distinct (Fig. 20). However, further subclustering was determined by sampling date and not by sampled habitat (Fig. 19).

The MRPP test showed that for both lakes the particle-associated BCs were significantly different from the free-living BCs (mean distance within groups = 0.58, mean distance between groups = 0.79). The BCs appeared more similar within the free-living fraction (LB: $\delta = 0.41$, LZ: $\delta = 0.57$) than within the particle-associated fraction (LB: $\delta = 0.63$, LZ: $\delta = 0.71$). The significance of the delta scores based on 999 permutations was 0.001.
Fig. 19. Dendrogram of Hellinger-transformed ARISA data of free-living BCs. Water of Lake Brienz (LB, triangles) was sampled in May and September 2009 and of Lake Zug (LZ, circles) in March and October 2009 based on T and O₂ profiles (see Fig. 6). Symbol colors indicate grouping by season and water layer, i.e., for LB epi- and hypolimnion and for LZ, oxic, oxycline, and anoxic water layers. Hierarchical cluster analysis was performed using average linkage, and Bray–Curtis distances were calculated from ARISA peak abundance.
4.4 Results

Fig. 20. Dendrogram of Hellinger-transformed ARISA data of particle-associated BCs. Water of Lake Brienz (LB, triangles) was sampled in May and September 2009 and of Lake Zug (LZ, circles) in March and October 2009 based on T and O\textsubscript{2} profiles (see Fig. 6). Symbol colors indicate grouping by season and water layer, i.e., for LB epi- and hypolimnion and for LZ, oxic, oxycline, and anoxic water layers. Hierarchical cluster analysis was performed using average linkage, and Bray–Curtis distances were calculated from ARISA peak abundance.

4.4.2. Amino acids

AA concentrations are shown in Table 10. For both lakes and sampling dates, the protein AA concentrations decreased with water depth with a slight increase just above the sediments. For both lakes the most abundant protein AAs were the acidic
AAs Glu (up to 473 nmol l\(^{-1}\)) and Asp (up to 393 nmol l\(^{-1}\)) and the neutral AAs Leu (up to 309 nmol l\(^{-1}\)), Ala (up to 287 nmol l\(^{-1}\)), and Gly (up to 239 nmol l\(^{-1}\)). Maximum AA concentrations were measured for the 5 m water depth of LZ sampled in October. The protein AA concentrations of LZ were two- to ten-fold higher than for LB. Minimum concentrations (< 1 nmol l\(^{-1}\)) were measured for the sulfuric AA Met and the aromatic AA Tyr for both lakes and sampling dates.

As Ala could not be detected for the 10 m, the 150 m and the 240 m water depth of LB in September, the DI was not calculated for these samples (Fig. 21A). In May, the DI showed a trend towards lower values with depth, which would indicate a degradation of sedimenting material (Dauwe et al. 1999). For LZ, the DI did not follow a conclusive pattern (Fig. 21E). Therefore, the DI values were not used for further data interpretation.

The concentrations of the nonprotein AAs \(\gamma\)-aba and Orn were below the limit of detection for approximately half of the 40 water samples. The determined contribution of the nonprotein AAs to the AA pool were \(\leq 1\) mol\%, with the exception of Orn for LB September, which reached up to \(\sim 5\) mol\% in the 70 m water depth. Therefore, the concentrations of nonprotein AAs were not used for further data interpretation.

4.4.3. **Amino sugars**

As reported previously, for both lakes and sampling dates the most abundant AS was GlcN, followed by GalN (Carstens et al. 2012). The AS concentrations strongly declined below the zone of primary production. The GlcN concentrations of LZ ranged from 2.78 (October, 100 m) to 70.6 nmol l\(^{-1}\) (March, 5 m) and were lower in fall than in spring (Carstens et al. 2012, Köllner et al. 2012). The AS concentrations of LB were also lower in fall compared to spring and were roughly one order of magnitude lower than in LZ.

GlcN/GalN has been proposed as an indicator for organic matter degradation, as decreasing GlcN/GalN values were reported to go along with organic matter degradation (Benner and Kaiser 2003, Davis et al. 2009). We observed highest GlcN/GalN values in the surface waters and lower GlcN/GalN values for samples from deeper waters (Fig. 21B,F; see also Carstens et al. 2012), which would be in line with degradation.
Relative to AAs, ASs are more resistant to degradation. Therefore, the values for AAs/ASs are expected to decrease during decomposition in the water column (Dauwe and Middelburg 1998). For both lakes, the AAs/ASs values showed highest values in the surface waters (Fig. 21C,G). The AAs/ASs values also showed considerable variation by season in the hypolimnion of each lake.

4.4.4. Chlorin Index

For both lakes and sampling dates, the CI values increased with depth, indicating degradation of primary produced pigment related POM during
Impact of amino compounds and organic matter degradation state on the vertical structure of lacustrine bacteria

Comparing lakes, the CI values for LZ were not significantly higher than for LB except when only the values for the spring samplings were compared ($t$-test, $\alpha = 0.05$, $p = 0.01$).

4.4.5. Explanatory variables with the strongest influence on between- and within-lake variability of the bacterial community structure

In total, we assembled and evaluated 35 variables, which included concentrations of TOC, TN, TP, ions, alkalinity, pH, T, $O_2$, concentrations of ASs and protein AAs, ratios thereof and the CI to determine their influence on the BC structures. As a first step, PCA was performed on this initial set of environmental variables. The circle of equilibrium contribution identified TP, TOC, ions, alkalinity, $O_2$, and T as the variables contributing most to the ordination graph (Fig. 22). The two lakes were clearly separated, which reflects the high concentrations of ions, TOC, and TP of LZ and the high $SO_4^{2-}$ and $O_2$ concentrations of LB. The 5 m to 25 m and the 5 m to 15 m water layers of LZ sampled in spring and fall, respectively, segregated from the main LZ cluster (Fig. 22).

![Fig. 22. PCA on 35 environmental variables and circle of equilibrium contribution which highlights the variables contributing significantly to the ordination, i.e., variables that have vectors outside of the equilibrium circle. The first two axes represent 78.7% of the variance. For abbreviations used see Table 7. Water samples from Lake Brienz are symbolized by triangles and Lake Zug by circles. For color code see Fig. 19 or Fig. 20.](image-url)
The 5 m and the 10 m water samples from fall plot outside of the equilibrium circle. This is mainly due to the high AS and AA concentrations in the surface waters of LZ. Fig. 22 further illustrates the high degree of overall correlation between the various amino compounds measured, as well as their distinctness from bulk parameters such as TN and TOC.

Based on the observed collinearities between various amino acids and between various ions measured, the RDA was performed with a reduced set of 19 explanatory variables, TOC, TN, TP, NO$_3^-$, SO$_4^{2-}$, Ca$^{2+}$, alkalinity, O$_2$, pH, T, GlcN, ManN, GalN, MurA, Glu - as representative for all AAs -, GlcN/GalN, C/N, AAs/ASs, and the CI. The variables used for RDA and their corresponding abbreviations are listed in Table 7. As the anoxic communities of LZ followed a very different dynamic (Fig. 19 & Fig. 20), particular trends between the different BCs of the two lakes may be obscured in the RDA. In the following section mainly the results for the RDA of the oxic water samples are described. The RDAs including also the anoxic water samples are shown in Fig. 24.

The RDA models with the full set of 19 explanatory variables were significant (p < 0.001, no of permutations = 1000) for both the free-living ($r^2_{adj} = 0.70$) and the particle-associated community data ($r^2_{adj} = 0.70$). For further variable reduction and in order to create an efficient model from the most significant explanatory variables, we applied forward selection of constraints using the forward.sel function of the R package packfor (Dray et al. 2009) and VEGAN’S ordistep function (Oksanen et al. 2011). Both functions selected a reduced set of nine explanatory variables for the RDA of the fee-living BC data (Fig. 23A). The parsimoniuous RDA indicated that within-lake variability was best explained by the variables T, NO$_3^-$, and CI.

For the particle-associated BCs, the same nine explanatory variables as for the fee-living BCs and additionally pH were selected (Fig. 23B). pH was strongly correlated with axis 2, which indicates its influence on the seasonal variation of the particle-associated BCC. In contrast to the fee-living BCs, the CI only had a very weak impact on the particle-associated BCC. The separation of the particle-associated BCs of LZ and LB along the first axis and of the sampling seasons along the second axis was preserved in the RDA comprising also the anoxic water samples (Fig. 24B).
In the RDA plot with the free-living BCs including the anoxic water samples, the oxic samples of LB and LZ were grouped in closer proximity compared to the anoxic samples of LZ (Fig. 24A). The anoxic BCs formed an outgroup as already shown by cluster analysis (Fig. 19).
Reflecting the main differences between the two lakes (Fig. 22), the BCs of LB were always correlated to high $\text{SO}_4^{2-}$, $\text{O}_2$, and $\text{NO}_3^-$ concentrations, and the BCs of LZ were always correlated to high alkalinity, C/N, TOC, and TP concentrations (Fig. 23A,B & Fig. 24A,B).

Fig. 24. RDA triplots of Hellinger-transformed ARISA data of (A) free-living and (B) particle-associated bacterial communities and constraining environmental variables. The first two canonical axes explain 39.2% and 30.6% of the total variance of the free-living and the particle-associated bacterial community data, respectively. Water samples from Lake Brienz are symbolised by triangles and Lake Zug by circles. Color coding is identical to Fig. 19 and Fig. 20.
4.4.6. **Bacterial cell abundance**

The highest bacterial cell counts were detected in the epilimnion of LZ (Fig. 25). In LZ, in which the TOC concentrations were three- to four-fold higher compared to the oligotrophic LB (Table 9), the number of bacterial cells correlated significantly with the TOC concentrations (\(p < 0.05, n = 10, \text{Table 8}\)). For both lakes and sampling dates, the bacterial cell counts were also significantly correlated with T and alkalinity (\(p < 0.05, n = 10\)). For both lakes, the bacterial cell number correlated significantly with C/N and the degradation index CI (\(p < 0.05, n = 10\)), with higher cell numbers corresponding to less degraded organic matter. However, for LB in May, the bacterial cell counts correlated only weakly with the CI (\(r^2 = 0.36, p = 0.07, n = 10\)). We further found significant correlations (\(p < 0.05, n = 10\)) between the bacterial abundance and the concentration of all the detected protein AAs, except for Lys of LB in May and Ala, Gly, and Lys of LB in September. In Table 8 correlations are only shown for Glu as a representative of all AAs. Correlation of cell numbers with concentrations of ASs was less consistent. Only for LB in May, significant correlations were found between bacterial abundance and all of the detected ASs (Table 8). The bacterial cell counts also correlated significantly with the TOC concentration for this sampling date (\(r^2 = 0.72, p < 0.01, n = 10\)).

![Fig. 25. Bacterial cell counts in the water column of Lake Brienz (LB) sampled in May and September 2009 and in the water column of Lake Zug (LZ) sampled in March and October 2009.](image)
Table 8. Pearson correlation coefficient ($r^2$) and significance of correlation between bacterial cell counts and biogeochemical data measured in the water columns of Lake Brienz (LB) sampled in May and September 2009 and of Lake Zug (LZ) sampled in March and October 2009

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LB May</th>
<th>LB Sep</th>
<th>LZ Mar</th>
<th>LZ Oct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth</td>
<td>0.61**</td>
<td>0.50*</td>
<td>0.68**</td>
<td>0.18</td>
</tr>
<tr>
<td>TOC</td>
<td>0.72**</td>
<td>0.33</td>
<td>0.57*</td>
<td>0.85**</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>0.01</td>
<td>0.92**</td>
<td>0.53*</td>
<td>0.86**</td>
</tr>
<tr>
<td>O$_2$</td>
<td>0.00</td>
<td>0.71**</td>
<td>0.66**</td>
<td>0.19</td>
</tr>
<tr>
<td>pH</td>
<td>0.19</td>
<td>0.94**</td>
<td>0.56*</td>
<td>0.49*</td>
</tr>
<tr>
<td>T</td>
<td>0.55*</td>
<td>0.95**</td>
<td>0.76**</td>
<td>0.73**</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>0.71**</td>
<td>0.96**</td>
<td>0.52*</td>
<td>0.61**</td>
</tr>
<tr>
<td>GlcN</td>
<td>0.54*</td>
<td>0.61*</td>
<td>0.36</td>
<td>0.61*</td>
</tr>
<tr>
<td>ManN</td>
<td>0.46*</td>
<td>0.82*</td>
<td>0.39</td>
<td>0.52*</td>
</tr>
<tr>
<td>GalN</td>
<td>0.56*</td>
<td>0.38</td>
<td>0.42*</td>
<td>0.41*</td>
</tr>
<tr>
<td>MurA</td>
<td>0.47*</td>
<td>0.25</td>
<td>0.08</td>
<td>0.65*</td>
</tr>
<tr>
<td>CI</td>
<td>0.36</td>
<td>0.68**</td>
<td>0.77**</td>
<td>0.76**</td>
</tr>
<tr>
<td>C/N</td>
<td>0.57*</td>
<td>0.60**</td>
<td>0.53*</td>
<td>0.97**</td>
</tr>
<tr>
<td>GlcN/GalN</td>
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<td>0.01</td>
<td>0.28</td>
<td>0.45*</td>
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<tr>
<td>Glu</td>
<td>0.49*</td>
<td>0.71**</td>
<td>0.78**</td>
<td>0.69**</td>
</tr>
</tbody>
</table>

*p $< 0.05$

**p $< 0.01$

4.4.7. Multiple regression analysis

Multiple regression analysis was performed with the variables Glu, C/N, CI, GlcN, alkalinity, T, O$_2$, NO$_3^-$, TOC, and water depth, for which significant correlations of $r^2 \geq 0.60$ and a p-value of $\leq 0.05$ with the bacterial cell abundance were found for at least two sampling dates. Glu was chosen as representative for all AAs. Multiple regression analysis with these ten variables suggested best four-variable model TOC-NO$_3^-$-GlcN-CI and best three-variable models GlcN-CI-C/N and TOC-CI-Glu. The suggested variables TOC, NO$_3^-$, GlcN, CI, C/N, and Glu all contributed significantly to the models (p $< 0.01$).
Lake Brienz sampled in May and September 2009 and Lake Zug sampled in March and October 2009.

Impact of amino compounds and organic matter degradation state on the vertical structure of lacustrine bacteria

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>MurA (µg l⁻¹)</th>
<th>GalN (µg l⁻¹)</th>
<th>ManN (µg l⁻¹)</th>
<th>Ca²⁺ (mg l⁻¹)</th>
<th>Na⁺ (mg l⁻¹)</th>
<th>Cl⁻ (mg l⁻¹)</th>
<th>TOC (mg l⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>March 2009</td>
<td>0.61</td>
<td>2.09</td>
<td>1.21</td>
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<tr>
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<td>2.49</td>
<td>1.03</td>
<td>78.2</td>
<td>8.73</td>
<td>3.58</td>
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</tr>
<tr>
<td>October 2009</td>
<td>0.73</td>
<td>0.99</td>
<td>0.61</td>
<td>80.0</td>
<td>9.19</td>
<td>3.90</td>
<td>6.53</td>
</tr>
</tbody>
</table>

Table 9. Basic chemical and physical parameters and concentrations of particular amino sugars of Lake Brienz sampled in May and September 2009 and Lake Zug sampled in March and October 2009.

Impact of amino compounds and organic matter degradation state on the vertical structure of lacustrine bacteria.

### Sampling date

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Lake Brienz</th>
<th>Lake Zug</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 2009</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Des</td>
<td>Asp</td>
</tr>
<tr>
<td></td>
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<td>0.53</td>
</tr>
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<td></td>
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<tr>
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<tr>
<td></td>
<td>Asp</td>
<td>Thr</td>
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<td></td>
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<td>0.76</td>
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### Lake Brienz

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### Lake Zug

<table>
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<th>Sampling site</th>
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<td>October 2009</td>
<td>10</td>
<td>100</td>
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### Notes

- For abbreviations of individual amino acids, see the provided link.
- Concentrations are given in nmol l⁻¹ for Thr, Ser, Val, Leu, Ile, Asp, Lys, γ Orn, and in µmol l⁻¹ for the other amino acids.
- The data are presented for both lakes and both sampling dates.

### Conclusion

The concentrations of particulate amino acids in Lake Brienz and Lake Zug were analyzed in May and September 2009, and March and October 2009. The data show a general increase in amino acid concentrations with depth, particularly in Lake Zug. The amino acids Thr, Ser, Val, Leu, Ile, Asp, Lys, and γ Orn were analyzed, with Thr, Ser, Val, Leu, and Ile showing the highest concentrations. The amino acid concentrations varied significantly between the lakes and the different sampling dates.
4.5. Discussion

A lake water column is characterized by strong depth-related gradients, many of which are autocorrelated. This is due to the strong effect of underlying physical conditions, e.g., the thermal stratification of the lake, the downward movement of sedimenting material, and the limitation of primary productivity to the upper water layers. Hence, for the identification of the environmental variables that drive vertical bacterial dynamics, care has to be taken. In this study, we have used a stepwise reduction of variable complexity aided by forward selection to extract the set of environmental variables with the strongest influence on the BCC dynamics. Further, we analyzed both full and partial datasets, e.g., separate analysis of the oxic/anoxic water body, to confirm consistency of trends. Finally, we evaluated the probable causality of the considered variables.

CI was consistently found to be strongly associated with the within-lake variation of the free-living BCCs (Fig. 23A & Fig. 24A). This was the case in both lakes despite the contrasting nutrient and redox conditions. For both lakes and samplings, the CI increased along the water column towards the lake sediments (Fig. 21D,H; see also Carstens et al. 2012), indicating POM degradation of sedimenting material. The CI was established as a reliable and simple tool for the characterization of the diagenetic state of POM for marine and lake habitats (Meckler et al. 2004, Schubert et al. 2005, Bechtel and Schubert 2009).

CI was not by itself a particularly good predictor for bacterial abundance (Table 8), but multiple regression indicated that it contributes significantly to explaining the variance of the bacterial abundance in the lake water columns. CI may be a particularly good indicator for the influence of organic matter on the abundance and composition of bacterioplankton since it is based on the degradation of chlorophyll and thus directly linked to the main source of degradable organic matter in the studied lakes, i.e., phytoplankton. Similarly, Rösel & Grossart (2012) associated increased bacterial abundance following after phytoplankton spring blooms with higher abundance of algal detritus (Rösel and Grossart 2012).

The abundance of specific particulate AAs, in particular the nonprotein AAs \( \gamma \)-aba and Orn, which are the degradation products of protein AAs (Lee and Cronin 1982), GlcN/GalN and AAs/ASs performed less well as predictors for BCC dynamics in relation to POM degradation in the lake water column. High GlcN/GalN values in
the surface waters of both lakes reflect synthesis of GlcN (Fig. 21B,F; Carstens et al. 2012). The GlcN/GalN values declined below the zone of primary production. As ASs are more resistant to degradation than AAs (Dauwe & Middelburg 1998), the values for AAs/ASs were found to be highest for the lake epilimnia and stabilized at low values in the deep waters (Fig. 21C,G). The GlcN/GalN and the AAs/ASs patterns indicate that within the water column, the degradation dynamics of particulate amino compounds were mainly restricted to the surface waters.

However, significant correlations between the bacterial cell abundance and the abundance of particulate amino compounds were found in the water columns of both lakes. Particulate amino compounds are not only significant growth substrates for particle-associated BCs, which accomplish the hydrolysis of POM, but also for the free-living bacteria in the surrounding water profiting from the hydrolysis products (Grossart and Simon 1998a, Beier and Bertilsson 2011).

Other abiotic factors with a strong impact on the BCC were T and O₂. For LZ, the composition of free-living BCs varied according to the O₂ gradient (Fig. 19, Fig. 23A & Fig. 24). As expected, the anoxic hypolimnion of LZ harbored a unique BC (Fig. 19 & Fig. 20). As bacteria in this region will have to rely on anaerobic respiration and fermentation, it is to be expected that only few organisms with highly flexible metabolism will thrive both above and below the oxycline.

Eliminating LZ’s anoxic waters from the analysis, T was found the second-best explanatory variable associated with the within-lake variation of the free-living BCC, in particular for LB since LZ was not thermally stratified during the sampling in March (see Fig. 6C). The T effect probably reflects the physical and biological structure of the lake, rather than it being a direct influence by itself: warm and light zone of primary production in the surface waters versus dark and cold hypolimnion, the zone of detrital matter turnover. T has previously been demonstrated as a significant variable reflecting the variance between epilimnion and hypolimnion BCs (Shade et al. 2008).

In comparison to the free-living BCs, the particle-associated BCs were more variable in their composition and influenced by different environmental variables. The CI was replaced by pH as the best explanatory parameter for within-lake variation of the particle-associated BCC. pH has previously been identified as an important driver of biogeochemical transformations and as cause of shifts in lake BCC (Lindström 2000, Yannarell and Triplett 2005).
The stronger impact of seasonal dynamics on particle-associated BCs compared to free-living BCs is in good agreement with recently published studies on the dynamics of these bacterial groups in lakes located in Germany (Rösel et al. 2012, Rösel and Grossart 2012). The pronounced seasonal dynamics of particle-associated BCs were linked to their tighter coupling to phyto- and zooplankton as they found particle-associated bacterial species strongly correlated to algal species and zooplankton biomass (Rösel et al. 2012). In the lakes presented here, LZ’s zoo- and phytoplankton differed substantially between the spring and the fall sampling, both in biomass and composition (Köllner et al. 2012) while it remained more constant in LB. This may be reflected in the stronger separation of seasonal clusters for the particle-associated BCs of LZ (Fig. 23B). The influence of seasonal phytoplankton successions on the BCC was shown previously as different algal species are sources for different types of substrates utilized by the bacterioplankton (Lindström 2001, Crump et al. 2003, Eiler and Bertilsson 2007, Šimek et al. 2011).

The > 5 µm fraction probably also comprises the preferred food particle size of grazers like nanoflagellates and ciliates since smaller bacterial species appeared more stable against grazing pressure (Salcher et al. 2010). Therefore, BCs in the > 5 µm fractions are probably more closely associated with the seasonal dynamics of these biota.

The BCs in the two contrasting lakes selected for this study were clearly separated (Fig. 23A,B). Concentrations of TOC, TP, SO\textsubscript{4}\textsuperscript{2−}, NO\textsubscript{3}, O\textsubscript{2} and alkalinity best explained this distinction. Gradients of TOC together with primary productivity were suggested previously as fundamental determinants of freshwater BCC dynamics (Yannarell and Triplett 2004, Allgaier and Grossart 2006) as these parameters integrate over the sources of energy used by bacteria for growth. For LZ, which had three to four times higher TOC concentrations than LB, the number of bacterial cells correlated significantly with the concentrations of TOC also in the depth gradient (Table 8) and highly significantly with the concentrations of DOC (data not shown because for LB most values were below or close to the detection limit of 0.50 mg l\textsuperscript{-1}).

The application of PCR-based techniques has its limitations, e.g., the preferential and nonspecific amplifications of DNA fragments probably leads to a biased representation of the BCs when applying molecular fingerprinting tools such as ARISA (Suzuki and Giovannoni 1996, Wang and Wang 1997, Sipos et al. 2007).
However, ARISA was previously demonstrated to be a reliable method for exploring temporal and spatial dynamics of BCs in freshwater ecosystems (Fisher and Triplett 1999, Yannarell et al. 2003, Shade et al. 2008). Additionally, it was used for the determination of environmental driving forces determining freshwater BCC (Yannarell and Triplett 2004, 2005, Shade et al. 2010). Therefore, we feel confident that the determined shifts in ARISA profiles mirror the differences in BCC in the lakes studied here.

In conclusion, the present study shows that not only the availability (TOC, GlcN, AA), but also the degradation state of organic matter (CI) drive the abundance and composition of lacustrine bacterioplankton. In the present study, CI, pH, and T were identified as the main environmental factors that shape the BCC in the depth gradient of two contrasting deep freshwater lakes. Particle-associated BCs appeared to be more variable in their composition and more directly affected by seasonal dynamics. These findings fit well to previous long-term studies of particle-associated bacteria in temperate lakes. Thus, future studied on the environmental factors shaping the vertical dynamics of lacustrine BCs should be analyzed on separated bacterial size fractions. We further recommend to include the CI as a proxy for organic matter degradation state in future investigations on the ecology of lacustrine bacteria.
5. CONTRIBUTION OF BACTERIAL CELLS TO LACUSTRINE ORGANIC MATTER BASED ON AMINO SUGARS AND D-AMINO ACIDS

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accepted Geochimica et Cosmochimica Acta

Contributions of the authors:
The paper was written by Dörte Carstens. The sampling was done by Dörte Carstens and Krista Köllner. Dörte Carstens did the biogeochemical analysis and interpretation of data. Krista Köllner measured the cell counts and contributed to the interpretation of the data. Bernhard Wehrli added significant contribution to the interpretation of the data. Helmut Bürgmann significantly contributed to the conception of the study. Carsten Schubert significantly contributed to the conception, the design, and the interpretation of the presented study.
5.1. Abstract

Amino sugars (ASs), D-amino acids (D-AAs) and bacterial cell counts were measured in two Swiss lakes to study the contribution of bacterial cells to organic matter (OM) and the fate of ASs and bacterial amino biomarkers during OM degradation. Concentrations of individual ASs (glucosamine, galactosamine, muramic acid and mannosamine) in the particulate and total OM pools were analyzed in water-column profiles of Lake Brienz (oligotrophic and oxic throughout the entire water column) and Lake Zug (eutrophic, stratified and permanently anoxic below 170 m) in spring and in fall. Generally, carbon-normalized AS concentrations decreased with water depth, indicating the preferential decomposition of ASs. For Lake Brienz the relative loss of particulate ASs was higher than in Lake Zug, suggesting enhanced AS turnover in an oligotrophic environment. AS ratio changes in the water column revealed a replacement of plankton biomass with OM from heterotrophic microorganisms with increasing water depth. Similar to the ASs, highest carbon normalized D-AA concentrations were found in the upper water column with decreasing concentrations with depth and an increase close to the sediments. In Lake Zug, an increase in the percentage of D-AAs also showed the involvement of bacteria in OM degradation. Estimations of OM derived from bacterial cells using cell counts and the bacterial biomarkers muramic acid and D-AAs gave similar results. For Lake Brienz 0.2-14% of the organic carbon pool originated from bacterial cells, compared to only 0.1-5% in Lake Zug. Based on our estimates, muramic acid appeared primarily associated with bacterial biomass and not with refractory bacterial necromass. Our study underscores that bacteria are not only important drivers of OM degradation in lacustrine systems, they also represent a significant source of OM themselves, especially in oligotrophic lakes.

5.2. Introduction

In nature, bacteria are key drivers for organic matter (OM) cycling. In the oceans about 50% of the planktonic primary production is processed through the microbial loop (Ducklow 2000). Fresh OM has high carbon-normalized yields of amino acids (AAs), neutral sugars, and amino sugars (ASs) (Cowie and Hedges 1992; Benner and Kaiser 2003). During OM decomposition, heterotrophic bacteria
preferentially utilize these reactive organic components (Cowie and Hedges 1992, Wakeham et al. 1997, Amon et al. 2001). While AAs have been widely measured in marine and lacustrine systems (Lee and Cronin 1982, Dauwe et al. 1999, Rosenstock and Simon 2001), only a few studies in aquatic science focus on ASs (Benner and Kaiser 2003, Fernandes et al. 2006, Tremblay and Benner 2009), despite the fact that they are considered to play a significant role in the nutrient cycle of aquatic systems (Nedoma et al. 1994, Vrba et al. 1997, Benner and Kaiser 2003). An abundant biopolymer consisting of ASs is chitin, a homopolymer of N-acetyl-D-glucosamine. The total annual chitin production in aquatic environments was estimated to $2.8 \times 10^{10}$ kg chitin yr$^{-1}$ in freshwater systems and to $1.3 \times 10^{12}$ kg chitin yr$^{-1}$ for marine ecosystems (Cauchie 2002). Up to 10% of the marine bacterial community could be sustained by chitin (Kirchman and White 1999). Other sources of ASs are polysaccharides, glycoproteins, and glycolipids that are common to many organisms (Sharon 1965). During degradation of OM, the AS composition changes e.g. the ratio between glucosamine (GlcN) and galactosamine (GalN) decreases with progressing degradation (Benner and Kaiser 2003, Davis et al. 2009). Besides being actively involved in OM degradation, bacteria themselves build up new biomass. The bacterial cell wall polymer peptidoglycan comprises GlcN and muramic acid (MurA), an AS that is unique to bacteria. As peptidoglycan is recycled within 10-167 d after organism death (Nagata et al. 2003), MurA can serve as a valuable biomarker for living bacteria and recent bacterial necromass (Moriarty 1975, Benner and Kaiser 2003, Niggemann and Schubert 2006). Another group of organic molecules which mainly derive from bacterial membranes are D-amino acids (D-AAs) which can amount to 1-3% of the dry weight of bacterial cells (Salton 1994). By analyzing these biomarkers, the contribution of bacteria to the OM pool has been studied in marine environments (McCarthhy et al. 1998, Ogawa et al. 2001, Kaiser and Benner 2008), estuaries (Bourgoin and Tremblay 2010), and rivers (Tremblay and Benner 2009).

It is well known that redox conditions have a direct effect on bacterial metabolism and consequently on the transformation pathways for specific organic compounds (Sun et al. 2002). For instance, studies on OM degradation processes showed more rapid degradation under oxic than under anoxic conditions (Harvey et al. 1995, Nguyen and Harvey 1997, Hedges et al. 1999). Most of these studies report on incubation experiments and concentrate on the degradation of OM in marine systems; field studies in lacustrine systems are rare.
This study aimed to estimate the contribution of bacterial cells to the OM pool in lacustrine systems with different trophic level and to investigate whether relations exist between nutrient status of a lake, preservation conditions, and AS compositional changes during OM degradation. Therefore, two contrasting lakes (oligotrophic and fully oxic versus eutrophic, stratified, and anoxic) in central Switzerland were investigated. Concentrations of single particulate ASs (PAS), particulate D-AA, and total ASs (TAS) were analyzed in the water columns of Lake Brienz and Lake Zug in two seasons. Bacterial cell counts were compared to quantitative estimates on the contribution of bacterial cells to OM based on the bacterial biomarkers MurA, D-alanine (D-Ala), and D-glutamic acid (D-Glx).

5.3. Methods

5.3.1. Sampling sites

Lake Brienz is a peri-alpine lake located in the northern ranges of the Swiss Alps, with a surface area of 29.8 km² (Fig. 5). It has a maximum depth of 259 m and a volume of 5.2 km³. The catchment of the lake is drained by the two main inflows, the Aare and the Lütschine, which together transport an annual average of 300,000 tons of suspended material into Lake Brienz (Finger et al. 2006). In contrast to the particle load, the nutrient input is low, resulting in an oligotrophic state of the lake. Lake Brienz was sampled in the central part at position 46°43'18''N/7°58'27''E where the lake has a depth of 250 m in May and September 2009.

Lake Zug is a eutrophic sub-alpine lake in central Switzerland about 30 km south of Zurich. Its maximum depth is 198 m and the volume of the lake is 3.2 km³ with a surface area of 38.4 km². Samples were taken in March and October 2009 at the deepest point of the lake at position 47°6'1''N/8°29'4''E. The sampling site is located in the meromictic southern basin of Lake Zug, where permanently anoxic conditions prevail below 170 m (Mengis et al. 1997).

5.3.2. Sampling

Water samples were taken with Niskin bottles in ten depths distributed over the entire water column (Lake Brienz: 5, 10, 20, 30, 40, 70, 100, 150, 200 and 240 m; Lake Zug: 5, 10, 15, 25, 60, 80, 100, 130, 170 and 190 m). The samples were split into separate aliquots for microbial and geochemical analyses. Water samples for the
microbial analysis were filled into autoclaved glass bottles (1 L) and transported cool and in the dark to the laboratory. For the analysis of TAS, a sample aliquot was poisoned with saturated HgCl₂ solution and stored at -20 °C until further processing.

Particulate matter (POM) was sampled at the same water depths with *in situ* pumps (McLane) onto two stacked precombusted glass fiber filters (GF/F filters, 142 mm diameter) with a nominal pore size of 0.7 μm (Whatman) until filters were clogged. Between 23 to 45 L of water were filtered in Lake Zug, whereas for Lake Brienz between 27 and 106 L were filtered. The filters were frozen directly after sampling and kept at -20 °C until analysis.

A 95 μm double closing net (Bürgi and Züllig 1983) was used to take an integrated plankton sample from 0-100 m water depth. The plankton samples were preserved in 2% formaldehyde.

Profiles of temperature, oxygen and conductivity were taken at each sampling campaign with a CTD-profiler (Seabird SBE19).

Primary productivity was estimated using the ¹⁴C-bicarbonate incubation method (Steeman-Nielsen 1952). For 13 water depths between 0 and 25 m, 120 mL of water were inoculated with 15 μCi NaH¹⁴CO₃. The Duran bottles were then incubated in situ for 3 h between 11:00 and 14:00 CET at the according water depths where the samples had been recovered. Afterwards, the samples were processed following the acid bubbling method of Gächter and Marès (1979). Therefore, the samples were split into two aliquots and the radioactivity of the added inorganic carbon was measured in one aliquot directly with a liquid scintillation spectrometer (TRICARB, Packard). The ¹⁴C activity of the organic substances built up during incubation was measured in the second aliquot after removal of the non-incorporated ¹⁴C by acidification and bubbling. The carbon assimilation rate was calculated as the fraction of incorporated ¹⁴C multiplied by the total dissolved inorganic carbon, which was determined from alkalinity and pH measurements according to Rodhe (1958).

### 5.3.3. Chemical analysis of water samples

Total organic carbon (TOC) and nitrogen (TN) concentrations of the water samples were measured by high temperature catalytic oxidation using a Shimadzu TOC-V CPH / TNM1 analyzer with a measurement error of 0.2 mg L⁻¹ and a detection limit of 0.5 mg L⁻¹.
Total amino sugar (TAS) concentrations in the water were measured using the method of Kaiser and Benner (2000). Due to low TAS concentrations, it was necessary to pre-concentrate the water samples. Thus, 45 mL of the water samples were lyophilized and redissolved in 2 mL of 3 mol L\(^{-1}\) HCl (Merck). For hydrolysis, the samples were flushed with nitrogen and kept 5 hours at 100 °C. After adding the internal standard 3-amino-3-deoxy-glucose hydrochloride for quantification (prepared from 3-acetamido-3-deoxy-D-glucose, TRC), the samples were neutralized with an AG11 A8 resin (50-100 mesh; Bio-Rad) and desalted with an AG50 X8 resin (200-400 mesh; Bio-Rad) for GlcN, GalN and mannosamine (ManN). The clean-up procedure for MurA was performed separately with AG50 X8 resin (100-200 mesh; Bio-Rad). Subsequently, ASs were separated using a metal-free high performance liquid chromatography system (Jasco) with a PAD ED50 detector (Dionex), equipped with a gold working electrode and a pH Ag/AgCl reference electrode. A CarboPac PA1 column (250 mm × 4 mm inner diameter; Dionex) with a CarboPac PA1 guard column (50 mm × 4 mm inner diameter; Dionex) was used. For the separation of GlcN, GalN and ManN the eluent was 12 mmol L\(^{-1}\) NaOH under isocratic conditions with a flow rate of 1 mL min\(^{-1}\). The separation of MurA was conducted at the same flow rate but with 99.6 mmol L\(^{-1}\) NaOH and 100 mmol L\(^{-1}\) sodium acetate as eluent under isocratic conditions. After hydrolysis, ASs are in the deacetylated form. This is the form which was used for calculation of AS yields. As ASs occur in nature commonly in the acetylated form, the presented calculations might underestimate AS carbon by up to 25% (Benner and Kaiser 2003). The relative standard error of the measurement was <13% for all measured ASs. The plankton samples were analyzed with the same procedure.

5.3.4. **Chemical analysis of particulate organic matter**

For the particulate amino sugar (PAS) analysis, one quarter of each filter was hydrolyzed with 6 mol L\(^{-1}\) HCl for 10 hours at 100 °C under N\(_2\). Based on earlier experiments of Klauser (2007) this procedure gave highest yields. The hydrolyzate was then processed following a slightly modified method after Zhang and Amelung (1996) including a derivatization step according to Guerrant and Moss (1984) and with myo-inositol (Aldrich) as internal standard. 1 μL of the derivatized extract was injected into a GC system equipped with a flame ionization detector (HRGC 5160, Carlo Erba Instruments), a split-splitless injector and a VF-5 MS column (60 m, 0.25
mm inner diameter and 0.25 μm film thickness; Varian). The injector temperature was 250 °C and the temperature of the detector was 300 °C. Hydrogen was used as carrier gas with a flow rate of 2 mL min⁻¹. The following temperature program was used: from 120 °C to 200 °C at 20 °C min⁻¹, from 200 °C to 250 °C at 2 °C min⁻¹, and from 250 °C to 270 °C at 20 °C min⁻¹ held 10 minutes at 270 °C. For quantification standards of D-GlcN (Sigma), D-ManN (Aldrich), D-GalN (Fluka), MurA (Sigma) and myo-inositol (Aldrich) were derivatized and measured on the GC system. The PAS concentrations were normalized to the amount of filtered water. The relative standard errors of triplicate analysis were 3-7% for GalN and GlcN and 11-19% for ManN and MurA.

For the analysis of particulate D-AAs, one quarter of each filter was hydrolyzed with 6 mol L⁻¹ HCl for 20 hours at 110 °C under N₂. Prior to hydrolysis, L-norleucine (Sigma) was added as internal standard. After drying under vacuum at 40 °C, samples were redissolved in 0.01 mol L⁻¹ HCl and processed according to Popp et al. (2007). In brief, samples were purified by cation-exchange chromatography (Dowex 50W X8 resin, 200-400 mesh; BioRad) as described by Metges et al. (1996). After the purification step, samples were reacidified with 0.02 mol L⁻¹ HCl at 110 °C for 5 min. Samples were then esterified with acidified isopropanol (4:1 isopropanol:acetyl chloride; Sigma Aldrich and Fluka) at 110 °C for 60 min and derivatized with 3:1 dichloromethane:trifluoroacetic anhydride (LabScan and Fluka) at 100 °C for 15 min to form trifluoroacetic amino acid esters. After drying, further purification was achieved by redissolving the sample in 1:2 chloroform (Mallinckrodt):phosphate-buffer (KH₂PO₄ + Na₂HPO₄ (both Fluka) in nanopure water, pH 7), shaking it vigorously and transferring the chloroform phase into a separate vial. Afterwards, the acylation step was repeated. The derivatized samples were dried, dissolved in ethyl acetate (Merck) and analyzed on a GC system with a flame ionization detector (Shimadzu) and a Chirasil-L-Val column (25 m, 0.25 μm inner diameter and 0.12 μm film thickness; Varian) using the following temperature settings: injector 180 °C, detector 280 °C, oven program: 80 °C held 5 min, 3.5 °C min⁻¹ to 120 °C held 3 min, 4 °C min⁻¹ to 152 °C held 3 min, 5 °C min⁻¹ to 195 °C held 10 min. The flow rate was 0.5 mL min⁻¹. Known amounts of D-Ala and D-Glx (Sigma) standards were derivatized and measured for quantification. D-AA concentrations were corrected for racemization during hydrolysis following Kaiser and Benner (2005). They determined the hydrolysis-induced racemization of free AAs and
proteins. Average percentages of single D-enantiomers (%D = 100 × D/(D+L)) produced during acid hydrolysis of the L-enantiomers in those experiments were used for correction of natural samples and were the following for D-Ala and D-Glx: 1.2% and 2.0%, respectively (Kaiser and Benner 2005). The relative standard errors of measurement were 4% for D-Ala and 9% for D-Glx.

Chlorin concentrations were measured on the POM samples using the procedure described by Schubert et al. (2005). The ratio of the fluorescence intensity of the acidified sample to the fluorescence intensity of the non-acidified original extract represents the Chlorin-Index (CI), which serves as a proxy for OM freshness (Schubert et al. 2005). The value of the CI for fresh chlorophyll a is 0.2 and increases up to unity for highly degraded organic material.

5.3.5. **Bacterial cell counts**

Bacterial cells were counted using flow cytometric measurements as described by Hammes et al. (2008). Briefly, bacterial cells were stained with 10 μL mL⁻¹ SYBR Green I (1:100 dilution in dimethyl sulfoxide; Molecular Probes) and incubated in the dark for 15 min at room temperature. The measurements were carried out on a CyFlow Space instrument (Partec) with a 200 mW laser, emitting light at a fixed wavelength of 488 nm and a volumetric counting hardware. At a wavelength of 520 (±20) nm, green fluorescence was determined and red fluorescence above 630 nm. The flow rate was 200 mL min⁻¹. For data analysis the Flowmax software (Partec) was used. Samples were diluted with cell-free water so that the measured concentration was between 20 and 500 events sec⁻¹. The standard error of triplicate measurements was <10%.

5.4. **Results**

5.4.1. **Physico-chemical characterization of the water columns**

The water column of Lake Brienz was fully oxic in spring and in fall with values between 7.3 and 10.2 mg L⁻¹ (Fig. 26). TOC concentrations varied between 0.5 and 0.9 mg L⁻¹ in spring and in fall, showing highest values in the upper water layers (Fig. 26). The TN concentrations in Lake Brienz were below the detection limit of 0.5 mg N L⁻¹ throughout the water column in both seasons. In spring, the areal primary production was estimated at 22 mg C m⁻² h⁻¹, where the depth of maximum primary
productivity was at 5 m. For the fall sampling, the areal primary productivity declined to a value of 12 mg C m\(^{-2}\) h\(^{-1}\) with a maximal productivity at 2.5 m depth.

Water column profiles for Lake Zug revealed oxygen concentrations of 9.2 mg L\(^{-1}\) near the water surface and decreasing concentrations with water depth in spring and in fall (Fig. 26). Below 130 m, the water column was anoxic in both seasons. The estimated areal primary production in spring was 206 mg C m\(^{-2}\) h\(^{-1}\) and declined in fall (98 mg C m\(^{-2}\) h\(^{-1}\)). Maximum productivity was observed at 2.5 m depth for each sampling event. In the photic zone, highest TOC concentrations were measured with values of 2.4 mg L\(^{-1}\) in spring and 2.5 mg L\(^{-1}\) in fall (Fig. 26). Towards the sediments the values decreased to 1.9 mg L\(^{-1}\). TN concentrations were close to the detection limit in spring and below the detection limit in fall.

Values for the CI were increasing with water depth in both lakes and seasons (Fig. 27). In Lake Zug, CI values ranged between 0.3-0.6 and 0.3-0.4 in spring and fall, respectively. In contrast to Lake Zug, where no clear temporal pattern could be discerned, we observed for Lake Brienz systematically lower CI values in spring (0.2-0.4) than in fall (0.3-0.5).

Fig. 26. Total organic carbon (TOC, black circles) and oxygen concentrations (black line) in Lake Brienz and Lake Zug, spring and fall 2009.
Fig. 27. Profiles of GlcN:GalN ratios and Chlorin-Indices (CI) in the particulate matter of Lake Brienz and Lake Zug, spring and fall 2009.

5.4.2. Total amino sugar concentrations

In Lake Brienz, TAS concentrations of the unfiltered water were below the detection limit in all samples. TAS concentrations in Lake Zug (Table 11) were higher than in studied open marine systems e.g. Pacific Ocean; Benner and Kaiser (2003), but were similar, for example to values found in Lake Murray (South Carolina, USA; Kawasaki and Benner (2006)). Carbon-normalized TAS concentrations in Lake Zug were highest in the upper water layers (Fig. 28). In spring, the TAS yields decreased slightly with depth. For MurA yields in spring a strong decrease below 5 m water depth and a strong increase just above the sediments were observed. In fall, a marked decline below 15 m was measured for all ASs. The contribution of TAS to TOC was 0.9 to 1.6% in spring and 0.6 to 2.7% in fall (Table 11). GlcN was the most abundant AS, contributing 52-68% to the TAS pool, followed by GalN (31-45%). MurA accounted for 0.2-1.5% of TAS. The GlcN:GalN ratios of the unfiltered water had values of 1.2-2.2 in spring and 1.2-1.8 in fall pointing to a bacterial origin of the ASs and diagenetic alteration (data not shown).
### Table 11. Molar particulate and total amino sugar concentrations in Lake Zug, spring and fall 2009

<table>
<thead>
<tr>
<th>Depth (m)</th>
<th>TOC (μmol L⁻¹)</th>
<th>GlcN POM (nmol L⁻¹)</th>
<th>ManN POM (nmol L⁻¹)</th>
<th>GalN POM (nmol L⁻¹)</th>
<th>MurA POM (nmol L⁻¹)</th>
<th>%TOC as PAS</th>
<th>GlcN total (nmol L⁻¹)</th>
<th>ManN total (nmol L⁻¹)</th>
<th>GalN total (nmol L⁻¹)</th>
<th>MurA total (nmol L⁻¹)</th>
<th>%TOC as THAS</th>
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<td>1.25</td>
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</table>

**Abbreviations used:**
- TOC: total organic carbon
- POM: particulate organic matter
- GlcN: glucosamine
- ManN: mannosamine
- GalN: galactosamine
- MurA: muramic acid
- PAS: particulate amino sugars
- THAS: total hydrolysable amino sugars
- bd: below detection limit
Contribution of bacterial cells to lacustrine organic matter based on amino sugars and D-amino acids

5.4.3. Amino sugars in POM

The PAS concentrations were approximately one order of magnitude higher in Lake Zug than in Lake Brienz in both seasons (see Table 11 and Table 12). Lake Zug had particulate GlcN concentrations between 8.5 and 70.6 nmol L\(^{-1}\) in spring with the highest value in 5 m depth and the lowest value in 60 m depth. In fall, the concentrations were systematically lower with values between 2.8 and 35.6 nmol L\(^{-1}\). Also in Lake Brienz highest concentrations were found in the upper water layers (2.9 nmol L\(^{-1}\) in spring and 0.9 nmol L\(^{-1}\) in fall). Lowest concentrations were in both seasons 0.7 nmol L\(^{-1}\) in the lower water column. Similar to the TAS, the PAS carbon-normalized concentrations were highest in the upper water layers (Fig. 29). For both lakes and seasons, a steep decline in the PAS yields could be observed below 10 m water depth. In spring and in fall, we observed an increase in MurA concentrations close to the sediments in Lake Brienz. A similar feature was also observed in Lake Zug, but only during the spring sampling. In Lake Brienz, PAS comprised 0.01-0.2% of the TOC. For Lake Zug the contribution of PAS to the TOC pool was slightly higher (0.02-0.3% of the TOC, Table 11). Generally, GlcN was the most abundant PAS in all samples with yields between 1.2-18.7 nmol (mg C\(^{-1}\)) in Lake Brienz and between 2.8 and 35.6 nmol (mg C\(^{-1}\)) in Lake Zug. GlcN comprised 35-70 mol% of PAS in Lake Brienz and 56-80 mol% of PAS in Lake Zug. GalN represented the second largest PAS fraction followed by MurA, except for the lower water column of Lake Brienz in fall (below 100 m), in spring the 5 m sample in Lake Brienz and in fall the 5 m sample in Lake Zug where the MurA concentrations were higher than those of...
5.4 Results

Table 12. Molar particulate amino sugar concentrations in Lake Brienz, spring and fall 2009

<table>
<thead>
<tr>
<th>Depth (m)</th>
<th>TOC (umol L⁻¹)</th>
<th>GlcN POM (nmol L⁻¹)</th>
<th>ManN POM (nmol L⁻¹)</th>
<th>GalN POM (nmol L⁻¹)</th>
<th>MurA POM (nmol L⁻¹)</th>
<th>%TOC as PAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Brienz spring</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>8.4</td>
<td>1.0</td>
<td>2.6</td>
<td>2.9</td>
<td>0.19</td>
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<tr>
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<td>0.07</td>
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<tr>
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<td>0.7</td>
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<td>70</td>
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<td>0.5</td>
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<tr>
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<tr>
<td>5</td>
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<td>3.9</td>
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<td>2.2</td>
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<td>0.1</td>
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<td>0.02</td>
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<td>0.2</td>
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<td>0.2</td>
<td>0.4</td>
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<tr>
<td>240</td>
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<td>1.9</td>
<td>0.2</td>
<td>0.4</td>
<td>0.7</td>
<td>0.05</td>
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</table>

*Abbreviations used:* TOC, total organic carbon; POM, particulate organic matter; GlcN, glucosamine; ManN, mannosamine; GalN, galactosamine; MurA, muramyl acid; bd = below detection limit.

GalN. ManN was the least abundant AS. GlcN:GalN ratios ranged between 1.8 and 5.6 in Lake Brienz and between 2.0 and 6.6 in Lake Zug (Fig. 27). Highest GlcN:GalN ratios were generally observed in the upper water column, with decreasing values towards the sediments, with the exception of the 240 m sample in Lake Brienz in fall, which is most likely an outlier, as the GlcN:GalN ratio can not be explained by resuspension from sediments, which had a GlcN:GalN ratio of 1.9 at the surface (data not shown) and another source of GlcN can be excluded.

In Lake Zug, PAS contributed between 3 to 9% to the TAS concentration in spring, except for the 5 m water sample, where it contributed up to 18%. In fall, the relative contribution of PAS to the total AS pool was strongly reduced when compared to the spring profile (by ~50% for all water depths and by 80% in the surface water).
The AS compositions of the plankton >95 μm were quite similar in both lakes with comparatively high yields of GlcN (> 150 nmol (mg C)^−1, Table 13). GlcN:GalN ratios were 6.2 for Lake Brienz and 9.7 for Lake Zug.

### Table 13. Elemental and amino sugar composition of the plankton >95 μm

<table>
<thead>
<tr>
<th></th>
<th>C (%)</th>
<th>N (%)</th>
<th>GlcN (nmol/mgC)</th>
<th>GalN (nmol/mgC)</th>
<th>ManN (nmol/mgC)</th>
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<td>164.4</td>
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<td>Lake Zug</td>
<td>42.4</td>
<td>8.8</td>
<td>223.9</td>
<td>23.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*Abbreviations used: C, carbon content; N, nitrogen content; GlcN, glucosamine; GalN, galactosamine; ManN, mannosamine.*
5.4 Results

5.4.4. D-Amino acids in POM

In spring, the range of molar concentrations of D-Ala were similar in both lakes with values between 0.4 and 8.5 nmol L\(^{-1}\) in Lake Brienz and 1.2 and 6.4 nmol L\(^{-1}\) in Lake Zug (Table 14). Highest concentrations were found in the upper water layers. D-Glx concentrations were in both lakes lower with 0.1-0.9 nmol L\(^{-1}\) and 1.5-4.7 nmol L\(^{-1}\) in Lake Brienz and Lake Zug, respectively. D-AA concentrations decreased with water depth. In fall, the concentrations in the upper water layers were similar to the spring sampling, but in the lower water column concentrations were lower. Below 130 m, a subtle increase in D-Ala was observed in Lake Zug for the spring and fall sampling. This was also the case for the 240 m depth in Lake Brienz in spring. D-AA concentrations were below the detection limit in almost all of the fall samples of Lake Brienz (Table 14). The patterns of the molar L-Ala and L-Glx concentrations looked similar to those of the D-AAs with high concentrations in the upper water layers, decreasing concentrations below the zone of primary productivity and an increase just above the sediments. In spring, the L-AA concentrations were in the same range in both lakes but with higher concentrations in Lake Zug. In fall, the L-AA concentrations were almost one order of magnitude higher in Lake Zug than in Lake Brienz.

D-AA carbon normalized concentrations are illustrated in Fig. 30. For Lake Brienz in spring the pattern was similar to that of MurA. Also in Lake Zug the yields were highest in the upper water layers. In fall, a decrease in D-AA yields with depth.

![Fig. 30. Carbon-normalized particulate D-amino acid (D-AA) yields in Lake Brienz and Lake Zug, spring and fall 2009.](image-url)
and a slight increase above the sediments was observed. For the spring sampling there was no consistent trend with depth in Lake Zug. The percentage of D-AAs (%D = 100 × (D/(D+L)); with D: molar concentration of D-AAs and L: molar concentration of the corresponding L-AAs) was 2.4-8.5% for the spring sampling in Lake Brienz and in Lake Zug 1.9-9% and 1.1-4.9% in spring and in fall, respectively (Table 14).

### Table 14. Molar particulate D-amino acid and corresponding L-amino acid concentrations and percentages of D-AAs (%D) in Lake Brienz and Lake Zug, spring and fall 2009

<table>
<thead>
<tr>
<th>Depth (m)</th>
<th>D-Ala (nmol L⁻¹)</th>
<th>L-Ala (nmol L⁻¹)</th>
<th>D-Glx (nmol L⁻¹)</th>
<th>L-Glx (nmol L⁻¹)</th>
<th>%D</th>
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Abbreviations used: D-Ala, D-alanine; L-Ala, L-alanine; D-Glx, D-glutamic acid; L-Glx, L-glutamic acid; %D = 100 × (D/(D+L)); bd = below detection limit.

#### 5.4.5. Bacterial cell counts

In both lakes, bacterial cell counts were in the same order of magnitude (3.8x10⁸ - 2.9x10⁹ cells L⁻¹); however, Lake Zug exhibited higher counts. The highest bacterial abundances were detected in the epilimnion, i.e. the zone of primary production (Fig. 31). The bacterial cell counts decreased with water depth. In Lake Brienz surface water, bacterial cell counts were higher in spring than in fall. Between
100 m depth and the bottom of the lake the bacterial abundance was similar for both samplings. For Lake Zug, higher cell counts were measured in the upper water layers in fall. A sharp decline in bacterial cell counts within the thermocline was observed, especially in fall. Below the oxic/anoxic water interface the bacterial cell counts increased towards the sediments especially in fall.

Fig. 31. Molar MurA concentrations of particulate matter and bacterial cell counts in Lake Brienz and Lake Zug, spring and fall 2009. MurA concentrations for Lake Brienz and Lake Zug are plotted on different scales.

5.5. Discussion

5.5.1. Origin and transformation processes of amino sugars in the water column

GlcN and GalN were the most abundant ASs in the present study. This is in agreement with studies from other environments and OM sources, e.g. samples of different size fractions from the Atlantic and Pacific oceans (Kaiser and Benner 2009), the Amazon River system (Tremblay and Benner 2009), different soils (Zhang and Amelung 1996), marine algae, copepods and bacteria (Benner and Kaiser 2003). ASs can originate from several potential sources, which are distinct in their AS composition. Chitin-producing phyto- and zooplankton for example is characterized by high GlcN:GalN ratios (>14), with chitin being a polymer of GlcN (Benner and Kaiser 2003). In contrast, ratios smaller than 3 are indicative for heterotrophic bacteria (Benner and Kaiser, 2003). In our study, the GlcN:GalN ratios of the particulate matter in the upper water column were higher than 3 (Lake Brienz 4.0, Lake Zug 7.8), but lower than the ratios of the analyzed plankton (Lake Brienz 6.2, Lake Zug 9.7) indicating that the PAS had planktonic and bacterial sources. Another
Contribution of bacterial cells to lacustrine organic matter based on amino sugars and D-amino acids

reason of lower GlcN:GalN ratios in the particulate matter is biodegradation of the planktonic matter which coincides with decreasing GlcN:GalN ratios (Liebezeit 1993, Benner and Kaiser 2003). Based on the 1:1 ratio of GlcN and MurA in peptidoglycan (Schleifer and Kandler 1972) we calculated the percentage of GlcN derived from bacterial peptidoglycan (GlcN_{peptidoglycan} (%) = 100 - (c_{GlcN} - c_{MurA})/c_{GlcN} \times 100, with c_{GlcN} and c_{MurA} being the molar concentrations of GlcN and MurA). Based on this equation, in the uppermost water layer of Lake Brienz, bacterial cell walls contributed 34% of the particulate GlcN in spring and 25% in fall. In contrast, in Lake Zug these fractions of bacterial cell wall derived GlcN were smaller, 6% and 15%, respectively. However, the ratios of GlcN:GalN in the POM decreased with water depth and had values smaller than 3 in both lakes and seasons. This indicates a shift from a planktonic AS signature towards the signal of heterotrophic microorganisms and progressing diagenesis (Ogawa et al. 2001, Benner and Kaiser 2003, Kawasaki and Benner 2006). For Lake Zug also an increase in %D from the upper water layers to the anoxic zone was observed. An increase in %D with water depth was also found e.g. in the St. Lawrence Estuary by Bourgoin and Tremblay (2010) and indicates bacterial degradation of POM. In Lake Brienz no consistent trend in %D was observed. Progressive degradation of sinking OM is also indicated by an increase in CI values with water depth. During the fall sampling, degradation seemed to be enhanced in Lake Brienz, as the CI gradient was more pronounced (Fig. 27). At the same time the GlcN:GalN ratios were lower in the upper water column compared to spring, indicating a higher contribution of bacteria to the particulate matter and a direct link between bacterial build-up and organic matter freshness. In Lake Zug, a somewhat different pattern was observed: CI values in the upper water column were higher and GlcN:GalN ratios smaller in spring than in fall.

In contrast to the PAS composition, the TAS pool comprised a rather small proportion of MurA in Lake Zug. The contribution of particulate MurA to the total MurA concentration reached 45 to 98% in spring. Whereas particulate GlcN and GalN contributed only up to 23% and 6% to the total GlcN and GalN concentrations, respectively. This agrees with findings from Benner and Kaiser (2003) from the Pacific Ocean, where MurA yields were higher in POM than in DOM samples. Furthermore, Nedoma et al. (1994) could not detect MurA in the dissolved form in freshwater environments and Jørgensen et al. (2008) found only insignificant concentrations of dissolved MurA in Mono Lake, but high concentrations of 25-75
nM in the unfiltered water. These findings indicate that MurA is rather associated with particulate matter than dissolved in the water. Interestingly, the molar MurA concentrations were in some POM samples higher than GalN concentrations. These results differ from findings from Benner and Kaiser (2003) as they found low MurA concentrations compared to GalN in POM samples. However, also in Lake Lugano higher MurA than GalN concentrations were measured in some POM samples (Klauser 2007), thus, this could be a feature of lacustrine AS compositions.

5.5.2. Contribution of bacterial cells to the organic carbon pool in the water column

Higher bacterial cell counts in the eutrophic Lake Zug compared to the oligotrophic Lake Brienz are in accordance with a survey of Chróst and Siuda (2006) who studied 19 lakes along a trophic gradient from meso/oligotrophic to hypereutrophic status. They found higher bacterial abundances in lakes with higher trophic levels and a strong relation between primary productivity and bacterial production. This coincides with high bacterial cell counts in the water layers of primary productivity in Lake Brienz and Lake Zug. In Lake Zug, the bacterial cell counts increased below the oxic/anoxic interface, highlighting the suboxic/anoxic zone in the water column as a hot spot of microbial activity. An increase in bacterial abundance was also observed in deep anoxic water layers of Mono Lake and the Black Sea (Humayoun et al. 2003, Morgan et al. 2006, Jørgensen et al. 2008).

In order to estimate the proportion of OM derived from bacterial cells in the water columns, we used two different approaches – an estimate based on average carbon content per cell and an estimate based on MurA, D-Ala and D-Glx concentrations. First, we calculated the bacterial carbon by multiplying the bacterial cell counts with an average carbon content of bacterial cells obtained from the literature. Measured average carbon contents of bacteria vary with cell size, environment and species. For prokaryotic cells in aquatic environments an average carbon content of 14 fg C cell\(^{-1}\) was determined by Fagerbakke et al. (1996). In marine systems, the carbon content of bacterial cells is, on average, 12.4 fg C cell\(^{-1}\) for the open ocean and 30.2 fg C cell\(^{-1}\) for pelagic coastal marine bacteria (Fukuda et al. 1998). Estimating the bacterial contribution to OM in an estuary, Bourgoin and Tremblay (2010) used an average bacterial cell carbon content of 11 fg C cell\(^{-1}\) (determined by Kawasaki et al. 2008). Applying this smallest value of 11 fg C cell\(^{-1}\),
we obtained as a minimum estimation that 0.8-3% and 0.3-1.5% of the carbon in Lake Brienz and in Lake Zug, respectively, derived from living cells, with higher fractions in the upper water column and for the spring sampling (Table 15).

Estimating the contribution of bacteria to OM in natural environments is associated to numerous uncertainties considering the diversity of bacteria and variations in the reactivity of biomarkers (Tremblay and Benner 2009). Nevertheless, MurA has been used as a marker for living bacteria or rather recent bacterial necromass. Mimura and Romano (1985) determined a strong correlation between MurA and bacterial counts in marine water samples and concluded that MurA can be used as an indicator for bacterial biomass. D-Ala and D-Glx also have a bacterial origin and thus have been used to estimate bacterial contribution to the organic carbon pool (e.g. Tremblay and Benner 2009; Bourgoin and Tremblay 2010; Kawasaki et al. 2011). Hence, in a second approach to assess organic carbon derived from bacterial cells, we used the concentrations of MurA, D-Ala and D-Glx according to the following equation (assuming that all bacteria were retained by the filter):

\[
\text{Bacterial C} \,(\%) = \frac{\text{biomarker}_{\text{sample}}}{\text{biomarker}_{\text{bacteria}}} \times 100
\]

with \(\text{biomarker}_{\text{sample}}\) being the measured particulate carbon-normalized yields of MurA, D-Ala or D-Glx, respectively, and \(\text{biomarker}_{\text{bacteria}}\) an average carbon-normalized yield of the biomarker from bacterial cells given in the literature (Kaiser and Benner, 2008). Depending on the bacterial assemblage, the range of the average bacterial MurA content is rather large. Gram-positive bacteria have a thicker cell wall than Gram-negative bacteria and therefore higher MurA contents (Schleifer and Kandler 1972). The average MurA yield for marine bacteria is 12.6 nmol (mg C)\(^{-1}\) (Benner and Kaiser 2003). For soil and freshwater heterotrophic bacteria Kaiser and Benner (2008) determined a mean of 42.3 nmol MurA (mg C)\(^{-1}\). Using this value in our study, we estimated (Eq. 1) organic carbon fractions derived from bacterial cells in the range of 0.2-11% and 0.3-5% for Lake Brienz and Lake Zug, respectively (Table 15). For the estimation of bacterial carbon contribution based on D-AAs, carbon-normalized yields of D-Ala and D-Glx published by Bourgoin and Tremblay (2010) (based on data of Kaiser and Benner 2008 and Tremblay and Benner 2009) for soil and freshwater bacteria were used (92.2 nmol D-Ala (mg C)\(^{-1}\) and 61.4 nmol D-
Glx (mg C\(^{-1}\)). With this approach we obtained similar contributions of particulate bacterial matter to the TOC (Table 15). These estimates were in spring between 0.7 and 14.4\% for Lake Brienz and 0.6 and 3.5\% for Lake Zug. Highest estimates were obtained for the upper water layers. Generally, our estimates for the bacterial derived carbon fraction for the two studied lakes are lower than estimates from the ocean, where Kaiser and Benner (2008) could show that ~25\% of the POM and DOM have a bacterial origin. This discrepancy can partly be explained by different methods used and the uncertainties of the approaches. First of all, the POM in the study of Kaiser and Benner (2008) had a size of 0.1-60 μm. The POM we sampled with GF/F filters had a size of >0.7 μm. In an experiment with marine bacteria these filters let through on average 25\% of bacteria (Gasol and Morán 1999). Although we used stacked

| Depth (m) | Lake Brienz | | | | Lake Zug | | | |
|-----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|           | spring      | fall        | spring      | fall        | spring      | fall        | spring      | fall        |
|           | D-Ala       | D-Glx       | MurA        | cell counts | D-Ala       | D-Glx       | MurA        | cell counts |
| 5         | 14.4        | 0.8         | 11.0        | 2.7         | -           | -           | 3.9         | 2.5         |
| 10        | 10.7        | 1.9         | 7.2         | 3.0         | -           | -           | 2.6         | 1.6         |
| 20        | 2.9         | 0.4         | 2.7         | 2.9         | 0.4         | -           | 1.6         | 1.1         |
| 30        | 4.0         | -           | 3.3         | 2.4         | -           | -           | 2.6         | 1.3         |
| 40        | 1.7         | -           | 1.5         | 1.5         | -           | -           | 1.4         | 1.0         |
| 70        | 2.1         | 0.6         | 0.4         | 1.0         | -           | -           | 0.6         | 0.9         |
| 100       | 1.1         | 0.3         | 0.9         | 1.1         | -           | -           | 1.1         | 0.9         |
| 150       | 1.0         | 0.2         | 0.3         | 0.8         | -           | -           | 1.8         | 0.9         |
| 200       | 0.7         | 0.2         | 0.2         | 0.8         | -           | -           | 4.5         | 0.8         |
| 240       | 2.0         | 0.9         | 1.7         | 0.9         | -           | -           | 3.3         | 1.0         |
|           | D-Ala       | D-Glx       | MurA        | cell counts | D-Ala       | D-Glx       | MurA        | cell counts |
| 5         | 2.1         | 2.0         | 4.7         | 1.5         | 1.5         | 2.0         | 1.5         | 1.5         |
| 10        | 2.3         | 2.5         | 1.2         | 1.1         | 1.1         | 2.6         | 1.1         | 1.1         |
| 15        | 3.1         | 3.5         | 0.7         | 1.3         | 1.3         | 1.4         | 1.3         | 1.4         |
| 25        | 2.3         | 1.4         | 1.0         | 1.3         | 1.3         | 1.7         | 1.3         | 1.7         |
| 60        | 0.6         | 2.4         | 1.1         | 0.8         | 0.8         | 0.3         | 0.8         | 0.3         |
| 80        | 1.4         | 1.3         | 0.7         | 0.6         | 0.6         | 0.4         | 0.6         | 0.4         |
| 100       | 2.2         | 2.5         | 1.2         | 0.5         | 0.5         | 0.1         | 0.5         | 0.1         |
| 130       | 0.9         | 1.3         | 0.9         | 0.6         | 0.6         | 0.2         | 0.6         | 0.2         |
| 170       | 2.2         | 2.0         | 1.6         | 0.7         | 0.7         | 0.6         | 0.7         | 0.6         |
| 190       | 2.3         | 1.7         | 4.1         | 0.7         | 0.7         | 1.1         | 0.7         | 1.1         |

Abbreviations used: D-Ala, D-alanine; D-Glx = D-glutamic acid; MurA, muramic acid.

filters, which slightly reduced the pore size, a certain fraction of bacteria was not retained by the filters, leading to an underestimation of organic carbon derived from
bacterial cells. Another uncertainty of the biomarker approach is the assumption that the biomarker yields of cultured bacteria represent also natural bacterial assemblages (Tremblay and Benner 2009; Kaiser and Benner 2008). By the same token, different bacterial cells sizes were not considered and probably also led to an underestimation of the carbon fraction derived from bacterial cells, as for example bacterial cells are larger in anoxic waters (Cole et al. 1993). Moreover, our study represents a minimum estimation of bacterial derived organic carbon as we investigated only the bacterial carbon derived from bacterial cells; dissolved organic carbon released from bacterial cells e.g. during growth, viral lysis and grazing was not included. However, the close agreement between the here-presented estimates based on bacterial cell counts, D-AAs and MurA not only provides confidence with regard to our assessment, it also suggests that MurA is a marker for bacterial cells rather than for refractory bacterial detritus. This somewhat stands in contrast to findings from the deep ocean, where Benner and Kaiser (2003) found that 10-15% of the MurA in POM samples is associated with intact bacterial cells, while a large fraction of MurA appears to be related to bacterial detritus. An explanation for these differences might be the greater water depth of the sampling sites in the ocean and the longer residence times of the particulate matter in the water column.

5.5.3. Effects of trophic status and redox conditions on amino sugar transformation and bacterial contributions to the organic carbon pool

The different nutrient levels of the studied lakes are reflected in a 10-fold higher primary productivity in the eutrophic Lake Zug (in spring 206 mg C m$^{-2}$ h$^{-1}$) compared to the oligotrophic Lake Brienz (in spring 22 mg C m$^{-2}$ h$^{-1}$). Higher productivity resulted in higher absolute PAS concentrations in Lake Zug compared to Lake Brienz. However, PAS yields had comparable values. Similar AS composition in the upper water layers probably derived from similar plankton communities as in both lakes the zooplankton was dominated by cladocernas and calanoid copepods and in fall diatoms were in both lakes the predominant phytoplankton species (Köllner et al. 2012). Below the zone of primary production, the biomass is degraded and the AS composition has a bacterial imprint (e.g. GlcN:GalN ratios). In Lake Brienz, the carbon-normalized PAS decrease from the surface water to the deep water column was greater than in Lake Zug. The contribution of PAS to the TOC pool, from surface to bottom waters, decreased by 83% and 72% in spring and by 47% and 42% in fall.
for Lake Brienz and Lake Zug, respectively. This finding suggests a more pronounced degradation of the AS pool and a higher turnover of OM under oligotrophic conditions. This observation may not only be restricted to ASs. Moreover, enhanced degradation of particulate fatty acids and neutral lipids under oligotrophic conditions in Lake Brienz compared to the eutrophic Lake Lugano were reported by Bechtel and Schubert (2009). The more enhanced degradation in oligotrophic systems might be due to smaller particles sizes in oligotrophic systems and therefore longer residence times in the water column (Stable 1984).

Interestingly, in both lakes the amino sugar yields stayed rather constant below the epilimnion (<30 m depth in Lake Brienz and <15m in Lake Zug). Hence, an immediate effect of changing redox conditions in the Lake Zug water column on the PAS composition was not observed. This could be explained by a short residence time of the particles in the anoxic zone or preservation of the OM under anoxic conditions. Another reason for the absence of a decrease in AS yields could be the presence of chemotrophic bacteria which built up new bacterial biomass during e.g. denitrification and therefore counterbalance the loss of ASs during degradation or directly use the ASs. However, the influence of redox conditions on the degradation of ASs has to be studied in more detail, as in our study only three samples in each season were taken from the anoxic water column.

A slight increase in AS, L- and D-AA concentrations, %D and cell counts right above the sediments was measured in both lakes and seasons which might derive from resuspension of sediments as a result of the formation of a benthic boundary layer (BBL). In these layers, which originate from intense mixing associated with seiching motions above the sediments, the concentration of organic particles is higher than above the BBL and serve therefore as zones of biogeochemical transformations (Gloor et al. 1994).

It has been shown in studies from lakes and reservoirs that in oligotrophic systems microbial biomass and heterotrophic microbial activity are relatively more important than in eutrophic systems (Biddanda et al. 2001, Cotner and Biddanda 2002, Caston et al. 2009). This can be explained by a better adaptation to low nutrient availability and a closer connection of autotrophic and heterotrophic processes in the euphotic zone (Cotner and Biddanda 2002). We also observed that bacteria, overall, comprise a slightly greater proportion of the OM in the oligotrophic Lake Brienz than in the eutrophic Lake Zug. This is also consistent with the GlcN:GalN ratios, which –
especially in fall – are lower in Lake Brienz than in Lake Zug. Furthermore, the fraction of GlcN derived from peptidoglycan is larger in Lake Brienz than in Lake Zug. These findings combined indicate enhanced transformation and therefore higher utilization of the primary produced organic carbon by bacteria in the oligotrophic Lake Brienz.
6. CONCLUSIONS AND OUTLOOK

This thesis elucidates the role of bacteria as degraders, but also as significant contributors of particulate organic matter in two lakes of contrasting trophic statuses. The focus was set on the bacterial degradation of the biopolymer chitin, which is synthesized in large amounts in aquatic ecosystems. Below, the main findings are summarized and aspects for further investigations are suggested.

6.1. Sites and significance of chitin hydrolysis

In chapter 2, the sites of chitin production (zooplankton) and the sediments were identified as the main sites of chitin hydrolysis. In the lake water columns, no chitinase activity could be measured. However, significant chitinase gene (chsA) concentrations were detected in the lake waters, in particular in the oligotrophic water column of Lake Brienz. In Lake Brienz with its unusually low primary production (< 10 g m\(^{-2}\) algal biomass), chitin appears to have a higher significance as growth substrate as other easily degradable substrates are scarce in contrast to eutrophic Lake Zug. This finding is supported by the results of a study conducted within this thesis, in which surface water samples of Lake Brienz, meso-oligotrophic Lake Lucerne, and Lake Zug were enriched with crab shell chitin (Wunderlin 2009). The maximum chsA concentrations, which were detected after two weeks of incubation, were measured for Lake Brienz, followed by Lake Lucerne and were lowest for Lake Zug. In order to test if bacterial communities of nutrient depleted lakes in general show a higher potential of chitin hydrolysis, future studies on the degradation of lacustrine chitin should include additional lakes of different trophic statuses.

In addition to significant correlations between parameters of chitin degradation (chsA gene abundance, chitinase activity) and carbon, nitrogen, and glucosamine (GlcN) concentrations in the sediments, in Lake Brienz’s water column the chsA concentrations correlated significantly (P < 0.01, n=10) with concentrations of GlcN at both sampling dates. In the water column of Lake Zug, for which chsA gene
Conclusions and outlook

abundance could only be detected for the surface waters, high chiA gene abundance was correlated with detected GlcN maxima on the respective sampling date. However, another significant source of GlcN is the bacterial cell wall polymer peptidoglycan. If measures of chitin turnover are related to the abundance of its monomer GlcN, it is suggested that bacterial derived GlcN is estimated on the basis of the 1:1 ratio of GlcN and muramic acid in peptidoglycan. Additionally, methods to quantify chitin in an aquatic ecosystem should be evaluated and the results of both approaches compared. For instance, the method developed by Montgomery and Kirchman (1994), which uses fluorescently or radioactively labeled wheat germ agglutinin, which binds to three consecutive N-acetylglucosamine residues, could be tested.

The lack of chitinase activity but detectable chiA abundance in the water samples of both lakes would indicate that free-living bacteria profit from the chitin hydrolysis products than being actively involved in the hydrolysis of chitin. This conclusion is based on the assumption that a bacterial species, which possesses the genetic tools for chitin hydrolysis (chiA) is also able to assimilate the products of chitin hydrolysis. The very low chiA copy numbers detected in the > 5 µm water fractions (see results in chapter 2) could point to a very small guild of chitinolytic bacteria associated with this particulate fraction, which releases chitin hydrolysis products in the surrounding waters of which the free-living bacterial communities could profit. In order to test for chitinase active particle size fractions in the water, future investigations should assay additional size fractions similar to Hoppe (1983). Furthermore, chitinase positive size fractions should be subjected to DNA sequencing to characterize the chitinases associated with the particulate fractions.

6.2. Bacterial chitinases

In chapter 3, the group of bacterial chitinases, which was found most abundant in the epi- and hypolimnic waters of Lake Brienz was not detected for the chitinase positive zooplankton (> 95 µm). This finding would point to planktonic bacteria not associated with chitin particles profiting from the chitinolytic activity of a different bacterial population. Such a commensal lifestyle was recently proposed for planktonic Actinobacteria (Beier and Bertilsson 2011). In contrast, in Lake Zug, the same bacterial chitinase associated with chitinase-active sites was also abundant in the
waters, which could indicate that in Lake Zug, the particle-associated chitinolytic bacteria sustain their free-living non-chitinase-active subpopulation. Such a ecological strategy was observed for cells of a strain of the marine bacterium *Pseudoalteromonas* sp. (Baty et al. 2000). Further research is needed to evaluate the distinct ecological role of particle-associated chitinolytic bacteria in lakes of contrasting trophic statuses.

In general, for both lakes and diverse lake habitats (zooplankton, water, sediment), a restricted number of bacterial chitinases, which were assigned to apparently rare members of the bacterial communities was detected. It can be assumed that their presence is essential for the functional trait of chitin hydrolysis in freshwater lakes. In order to detect additional significant chitinases in future studies, different subsets of primer pairs specific for particular groups of bacteria, which were detected via 454 pyrosequencing of the 16S rRNA gene, could be designed and applied. For instance, in the water column of Lake Brienz, apart from the surface waters, the *chiA* gene abundance peaked in the 150 m and the 200 m water layer sampled in May and September, respectively. In these water layers, *Chitinophagaceae* were substantially more abundant compared to the other lake habitats. Members of this family could be significant for the hydrolysis of detrital chitin in Lake Brienz.

Some chitinases detected in the surface water samples of Lake Brienz and Lake Zug were assigned to known chitinases of β-Proteobacteria (*Janthinobacterium lividum*). Bacterial species, which became abundant during chitin enrichment experiments conducted within this thesis (Wunderlin 2009) were also identified as β-Proteobacteria belonging to the *Burkholderiales* (*Comamonadaceae*) and *Neisseriales* (*Neisseriaceae*) (Köllner unpublished data). They became abundant due to the chitin enrichment and were not detected in the initial bacterial community by DGGE fingerprinting (Wunderlin 2009). This would support that rare members of the bacterial community are responsible for the hydrolysis of freshwater chitin as discussed above. However, chitinases from *Comamonadaceae* and *Neisseriaceae* could not be detected in the zooplankton samples (chapter 3) although they were detected as members of the total bacterial communities associated with zooplankton - in the zooplankton sample of Lake Brienz, *Comamonadaceae* made up 20% of the sequences. In a study on the bacterial diversity associated with freshwater copepods, β-Proteobacteria (*Neisseriaceae, Comamonadaceae, Oxalobacteraceae*) were the most abundant bacteria next to *Bacteroidetes* (Grossart et al. 2009). In order to examine if these groups of bacteria are significant for the degradation of lacustrine
zooplankton chitin, future studies could include laboratory experiments similar to Tang et al. (2006). The bacterial phyla which colonize and decompose the zooplankton carcasses could be identified by combining microscopic and molecular analytical methods. Application of MAR-FISH using specific probes targeting the identified bacteria would show the consumption of chitin or products of chitin hydrolysis. This approach would also provide quantitative estimates on the abundance of these bacteria in the lakes under study and was successfully applied previously (Cottrell and Kirchman 2000, Beier and Bertilsson 2011).

The predominant chitinase detected for the water samples of Lake Brienz could be identified as a known chitinase from *Stenotrophomonas maltophilia*, whereas the predominant chitinase in Lake Zug appeared to present a novel bacterial chitinase lineage (chapter 3). In future experiments, the cultivation of chitinolytic bacteria from Lake Zug on chitin extracted from autochthonous zooplankton and analysis of the associated chitinases could probably identify this chitinase lineage. Additionally, water samples from the hypolimnion of Lake Brienz, for which high *chiA* gene abundance was detected via quantitative PCR (chapter 2), could be subjected to enrichment experiments. As diatoms could also be a significant source of freshwater chitin (Smucker 1991), it could also be tried to culture chitinolytic bacteria on chitin extracted from diatoms. With this experiment it could be tested if the chitinolytic consortia on diatom chitin are 1) different from those determined on zooplankton chitin and/or 2) grow faster as this chitin type is not cross-linked to other structural components and represents the more open β-form (compared to the tightly packed α-chitin of crustaceans). In order to identify the bacterial species grown on these autochthonous chitin sources, samples could be subjected to cloning and sequencing.

6.3. **Organic matter degradation indices and bacterial dynamics**

In both lakes, the free-living (> 0.2, < 5 µm) and the particle-associated (> 5 µm) total bacterial communities showed structural shifts along the lake water columns (chapter 4). It was hypothesized that these vertical shifts are accompanied by shifts in the composition and degradation state of particulate organic matter. Whereas amino compound based degradation indices performed less well as indicators for the bacterioplankton dynamics, the Chlorin Index was identified as one of the main predictors for the abundance and composition of bacterioplankton in the water.
columns of both lakes. The Chlorin Index is a degradation index based on the
degradation state of chlorophyll, and, therefore, directly linked to a significant source
of growth substrates for lacustrine heterotrophic bacteria, i.e., organic matter
produced by phytoplankton. Thus, the CI appeared as a good indicator for the
succession of bacterial communities in lakes.

However, for the analysis of the CI and the individual particulate amino
compounds, organic matter was collected on filters with a pore size of 0.7 µm. In
future investigations, shifts in the community structure and abundance of bacteria
should additionally be analyzed for the 0.7 µm water fraction in order to derive
correlations between bacterioplankton dynamics and the quality and quantity of amino
compounds detected for this size fraction. To follow the seasonality of organic matter
degradation patterns in relation to seasonal dynamics of the bacterial community
structure, the lakes should be sampled more frequently. In particular, the dynamics
close to chlorophyll $a$ or primary productivity maxima and afterwards should also be
determined to record maxima in production of organic matter and the fate of detrital
matter thereafter. The response of the bacterial community structure could be
reflected by appearing and disappearing of particular bacterial species which can be
identified by cloning and sequencing.

6.4. Bacterial contribution to organic matter

Applying bacterial cell counts and the bacterial amino biomarkers muramic
acid and D-amino acids, the proportion of bacterial derived organic carbon was
consistently higher in the oligotrophic water column of Lake Brienz than in the
eutrophic water column of Lake Zug. Higher turnover rates of particulate amino
sugars in Lake Brienz point to a more pronounced degradation of particulate organic
matter from primary production in Lake Brienz than in Lake Zug. These findings are
in accordance with relatively higher bacterial metabolism and higher fractions of
particulate carbon, nitrogen and phosphorus as bacterial biomass in oligotrophic
compared to eutrophic freshwater systems (Biddanda et al. 2001, Caston et al. 2009).
This can be explained by a better adaption of bacteria to low nutrient availability, for
instance, bacteria can outcompete phytoplankton for inorganic nutrients, due to their
large cell surface to volume ratios (Currie and Kalff 1984), whereas in eutrophic
systems, bacteria were shown to underlie a greater grazing pressure and increased viral mortality (Sanders et al. 1992, Weinbauer et al. 1993).

However, the biomarker content differs between bacterial species and cell sizes and also between cultured and natural bacteria (Kaiser and Benner 2008). Therefore, future studies should include microscopic analysis (DAPI, FISH) or special flow cytometry applications to determine the size and shape of bacterial cells, which could increase the accuracy of the estimated bacterial derived organic matter (Hammes and Egli 2010).

In conclusion, higher potential of chitin hydrolysis and higher contribution to the carbon and nitrogen budget in oligotrophic Lake Brienz than in eutrophic Lake Zug pointed to a higher significance of chitin as growth substrate in systems where more readily available organic substrates are scarce. Supportingly, higher turnover rates of particulate amino sugars were detected in Lake Brienz. A restricted number of bacterial chitinases, which were distinct between the two lakes, appeared to be responsible for the functional trait of chitin hydrolysis. The higher proportion of bacterial derived organic carbon underscores the higher significance of bacteria for nutrient and carbon flow in oligotrophic freshwater systems.

Future studies including additional lakes of contrasting trophic state could reveal the higher importance of heterotrophic bacteria for the recycling of recalcitrant organic matter in oligotrophic systems as a general feature. In particular, it could be investigated if chitinolytic bacteria are generally higher abundant in oligotrophic than in eutrophic lakes and if similar bacterial chitinases are distributed between systems of similar trophic state.
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ACKNOWLEDGMENTS

I would like to thank all the people who supported me during my time as a PhD student. Without you this thesis would not exist!

First I would like to express thanks to my supervisors Dr. Helmut Bürgmann and Prof. Josef Zeyer. Helmut, thanks for giving me the opportunity to work at this extraordinarily beautiful place Kastanienbaum, your confidence, and “open doors”. Thank you Sepp for caring about the progress of my work, your good advice and correcting and improving my manuscripts.

I am grateful to Prof. Hans-Peter Grossart for co-examination of this thesis.

I would like to thank my project partners Carsten Schubert and Dörte Carstens for the good collaboration. Thanks to Carsten for correcting and improving my manuscripts and the special project meetings. In particular, I want to thank Helmut and Carsten for selecting Dörte and me for this challenging interdisciplinary project. It was a great pleasure to work with you Dörte! Thank you for all the discussions, your sense of humor and optimism!

I am grateful to all the people who helped me in the field: Michael Schurter, Alois Zwyssig, Francisco Vazquez, Tina Wunderlin, Torsten Diem, Gijs Nobbe, Leticia Stojkovski, Mathias Kirf, and Beat Kienholz.

In particular, I would like to thank Francisco Vazquez for his great support in the field and in the lab, his outstanding expertise and commitment and his friendship.

I am grateful to Esther Keller for analyzing the zoo- and phytoplankton samples from Lake Zug. Frederik Hammes is acknowledged for support with flow cytometry analyses. Thanks to Stefan Zoller from the Genetic Diversity Center ETH Zurich for helping with bioinformatics. I am grateful to Markus Zeh and Katrin Guthruf from the Environmental Agency of Canton Bern for providing the plankton monitoring data of Lake Brienz. Peter Keller from the Environmental Agency of Canton Zug is acknowledged for providing monitoring data from Lake Zug.

I would like to thank the Aua Lab and Ruth Stierli for the basic chemical analysis.

My special thanks are extended to the great secretaries’ team Eliane Scharmin, Patricia Achleitner, Luzia Fuchs, and Nadja Pepe for their administrative support.

I thank the Swiss National Science Foundation for funding this study.

Thank you Lawrence, Jeff, Oliver, and Dörte for cooking together and the humorous lunch breaks. Thank you Chregu for sharing the coffee breaks with me and the interesting discussions.

I am very grateful to my family and friends for their support, their sympathetic ear and motivation during my PhD work.