Bacterial Degradation and Use of Chitin in Aquatic Habitats

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Dissertation presented at Uppsala University to be publicly examined in Ekmansalen, Evolutionsbiologisk Centrum, Norbyv. 18 D, Uppsala, Friday, November 5, 2010 at 10:00 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

Chitin belongs to the most abundant biopolymers on earth where it has an important role as a structural element in crustaceans, insects, fungi and some phytoplankton. Missing evidence for long-term accumulation of chitin in nature implies fast turnover and as chitin is composed of aminosugar subunits it holds central roles in both carbon and nitrogen cycles. The aim of this thesis was to contribute to a better understanding of organic matter cycling by learning more about the diversity, function and ecology of bacteria that degrade chitin. A metagenome-enabled study of the spatial distribution of chitinolytic bacteria in aquatic ecosystems identified salinity as the major environmental factor for shaping their community composition. To address the role of alternative environmental variables controlling chitinolytic communities, a temporally resolved study was completed in a dimictic freshwater lake. Pronounced seasonal change in the indigenous chitinolytic community was observed and parallel measured environmental parameters pointed to the availability and crystalline form of chitin as significant controlling factors. The different ecological niches occupied by microbes that utilize chitin for growth were studied in an experimental study. Single-cell quantification of chitinolytic cells and cells incorporating chitin hydrolysis products suggested that commensal use of chitin hydrolysis products without simultaneous chitinase activity could be an important ecological strategy in freshwater bacterioplankton communities. Members of the ubiquitous and often quantitatively dominant group of freshwater Actinobacteria Ac1 were identified as particularly active in this “cheater” lifestyle. Further experiments based on artificially created gradients in bacterial diversity demonstrated the importance of specific bacterial populations and community composition rather than overall community richness in controlling more specific functions such as chitin and cellulose degradation. To conclude, results of this thesis provide insight into the biogeography, niche-separation and species interactions of the functional community of chitin degraders and the influence of general bacterial diversity to the respective system functioning.

Keywords: Chitin, organic matter degradation, microbial ecology, functional guild

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“I could recount an endless number of stories about carbon atoms that (...) from tiny algae to small crustaceans to fish gradually return as carbon dioxide to the waters of the sea, in perpetual, frightening round-dance of life and dead; of others which instead attain decorous semi-eternity in the yellow pages of some archival document...”

(Primo Levi, The Periodic Table)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


II  Beier S., Jones C. M., Mohit V., Hallin S., Bertilsson, S. Global phylogeography of *chitinase* genes in aquatic metagenomes. *Manuscript.*


Paper V reprinted with permission from Nature Publishing Group
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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>16S rRNA</td>
<td>bacterial small subunit ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DON</td>
<td>dissolved organic nitrogen</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELF®97-GlcNAc</td>
<td>ELF®97- N-acetyl-D-glucosaminide</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl-D-glucosamine</td>
</tr>
<tr>
<td>(GlcNAc)₂</td>
<td>N,N’-diacetylchitobiose</td>
</tr>
<tr>
<td>MAR-CARD-FISH</td>
<td>microaudioradiography - catalyzed reporter deposition - fluorescence in situ hybridization</td>
</tr>
<tr>
<td>MUF</td>
<td>methylumbelliferyl</td>
</tr>
<tr>
<td>OTU</td>
<td>operational taxonomic unit</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>TRF</td>
<td>terminal restriction fragment</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>terminal restriction fragment lengths polymorphism</td>
</tr>
<tr>
<td>WC medium</td>
<td>Wright’s Chryptophyte medium</td>
</tr>
</tbody>
</table>
Particulate and dissolved polymeric substances are major components of aquatic organic matter (Middelboe et al. 1995; Kaplan et al. 2003) and can dominate the pool of carbon sources readily available for bacterial heterotrophs (Middelboe et al. 1995). Chitin belongs, along with cellulose (Gooday 1990a) and possibly also murein (Whitman et al. 1998; Kaiser et al. 2008), to a shortlist of highly abundant biopolymers with enormous global production rates. At the same time there are so far no reports of quantitatively significant long-term accumulation of chitin in nature (Tracey 1957; Gooday 1990a). Analysis of marine DON (dissolved organic nitrogen) even implies chitin-derived substances as a more labile DON component than amide-containing N-compounds (e.g. proteins), with assumed high turnover rates (Aluwihare et al. 2005). Despite the lack of long term chitin accumulation in nature, temporary accumulation of chitin may occur, e.g. during phases of high chitin production at certain times of the year (Miyamoto et al. 1991). Also fossils may occasionally contain traces of chitin, even if fast degradation of chitin has been reported also in the absence of oxygen. It was suggested that certain chitin-protein complexes, such as histidyl and catechol cross-linkages can stabilize chitin. Formation of such complexes might slow down or stop chitin decay if combined with strongly reducing embedding material like tuff deposits. (Stankiewicz et al. 1997; Stankiewicz et al. 1998; Flannery et al. 2001).

There are also studies suggesting that complexation with certain metals (e.g. mercury) may protect chitin from chitinolytic enzymes (Krantz-Rülcker et al. 1995).

Gooday (Gooday 1990a) estimated the annual production and steady state amounts of chitin in aquatic systems, roughly equal to $10^{10}$-$10^{11}$ metric tons. The occurrence of chitin is widespread among organisms, where it serves as a stabilization element. It is produced by several eukaryote species within the ciliates, flagellates, amoebae, chrysophyte algae, centrix diatoms, filamentous chlorophyte algae and oomycete fungi, e.g. in cellwalls, cystwalls or flotation spines. It is further a major structural cell wall or skeleton component of nearly all fungi and many invertebrate phyla or classes such as hydrozoa, nematodes, rotifers, brachiopods, annelids, mollusks and arthropods. In contrast, chitin is absent in vertebrate animals and higher plants. Within prokaryotes chitin has only been detected in the spores of Streptomyces. (Gooday 1990a; Gooday 1990b).

Major sources of
chitin in aquatic environments are planktic microcrustaceans and chitin containing diatoms that can reach high local abundances during bloom events

Structure of Chitin

Chitin is the polymer of (1→4)-β-linked N-acetyl-D-glucosamine (GlcNAc). The single sugar units are rotated 180° to each other and the disaccharide N,N’-diacetylchitobiose ((GlcNAc)_2) could be considered as the structural unit. Chitin occurs with varying degree of deacetylation and the transition to chitosan, which is the deacetylated form, is therefore hard to define. Chitin builds helices which assemble into microfibrils, and are stabilized by hydrogen bonds between the amine and the carbonyl groups. It can be classified into three different crystalline forms: in the α-form the microfibrils are arranged in antiparallel orientation. In the β-form, microfibrils are oriented in parallel whereas γ-chitin features a mixed formation of antiparallel and parallel-orientated microfibrils. These three types of chitin primarily differ in the degree of hydration, in the size of the unit cell and in the number of chitin chains per unit cell. With few exceptions, natural chitin occurs covalently or non-covalently bound to other structural units, such as proteins or glucans, which often, constitute more than 50% of chitin containing structures (Attwood et al. 1967; Schaefer et al. 1987; Merzendorfer et al. 2003). Chitin is a structural homologue to cellulose, with the difference that the latter is composed of glucose instead of GlcNAc subunits. Murein is the main polymer in bacterial cellwalls and is also considered to be a chitin homologue as it is composed of alternating (1→4)-β-linked GlcNAc and N-acetylmuramic acid units.

Figure 1. The structural subunit of chitin: diacetylchitobiose ((GlcNAc)_2).
Processes involved in chitin degradation

In accordance with the ubiquity of chitin in nature, chitin modifying enzymes are found in a broad range of organisms where they may serve as virulence factors in insect pathogens, defense mechanisms against fungal pathogens or morphogenesis in arthropods. Bacteria, or in some habitats also fungi, are believed to be the main degraders of chitin in natural ecosystems (Gooday 1990a). Moreover some archaea also possess chitinases (Huber et al. 1995; Tanaka et al. 1999; Gao et al. 2003).

Chitinolysis involves the initial cleavage of the β-(1→4) bond between the chitin monomers and is catalyzed by chitinases belonging to glycoside hydrolase family 18 or 19 proteins. The appearance of multiple chitinases within a single organism is frequently reported (e.g. Fuchs et al. 1986; Saito et al. 1999; Tsujibo et al. 2003). Lateral gene transfer, gene duplication and post-transcriptional protein modifications have been described as mechanisms causing this enzyme multiplicity among bacteria. Synergistic effects among different chitinase proteins (Hult et al. 2005) as well as contrasting efficiency in degrading different crystalline forms of chitin (Svitil et al. 1997) have been discussed as factors that could favor organisms containing multiple chitinase genes.

Chitinolysis is often considered to be the quantitatively most important process for chitin degradation, but also processes that include the initial cleavage of the functional groups of the chitin subunits are described. Examples of this is deaminasis and deacetylecis that transform chitin to chitosan, (Hillman et al. 1989; Gooday 1990b). The polymer degradation then proceeds by the action of chitosanases or cellulases. Chitin degrading processes in general, independent on the mechanisms involved, are called chitinoclastic.

Besides the enzymes interacting directly with the chitin substrate, a number of additional proteins are involved in sensing, chemotaxis and expression upregulation during bacterial chitin degradation.
Commensal use of chitin hydrolysis products

Initial microbial hydrolysis of polymers such as chitin has to take place outside of the cells, and therefore cause extracellular release of hydrolysis products. For some chitinolytic strains, efficient uptake systems will minimize the loss of hydrolysis products (Bassler et al. 1991). However, this is not a universal ecophysiological feature of chitinolytic organisms and it has been shown that within some bacterial strains, the majority of clonal populations can profit from extracellular hydrolysis products produced by a few individuals in the population (Baty et al. 2000). The chitin degrading machinery includes up to 50 different proteins (Keyhani et al. 1999; Li et al. 2004; Meibom et al. 2004), and multicellular cooperation with only some cells investing in the expression of those proteins seems as a reasonable strategy for efficient population level resource use. Overproduction of enzymatic activity observed on particulate material in aquatic systems (Smith et al. 1992; Kirchman et al. 1999) might be caused by this kind of multicellular interactions. On the other hand, also other populations of commensals or parasitic organisms might take advantage of the freely available hydrolysis products. The existence of bacterial strains that take up
chitin mono- or dimers, GlcNAc and (GlcNAc)$_2$ without being able to hydrolyze polymeric chitin has indeed been described (Kaneko et al. 1978; Keyhani et al. 1997). Recently, a parasitic relationship between a chitin degrader and a non-chitin degrading bacterium was studied (Jagmann et al. 2010). Still, little attention has so far been paid to the quantitative significance of such commensal or parasitic relationships in natural aquatic environments.
Aims

The overall aim of this thesis is to contribute to the understanding of organic matter cycling by learning more about the diversity, function and ecology of bacteria that degrade chitin. Chitin belongs to the most abundant biopolymers on earth, while missing evidence for accumulation implies enormous turnover rates.

Methodological advances have resulted in an enormous accumulation of knowledge concerning the appearance, diversity and distribution of bacteria. Recently developed methods have greatly improved our ability to directly link functionality to phylogenetic identity of microorganisms without relying on cultivation. I have applied phylogenetic based molecular approaches as well as single cell techniques to address the following major questions related to the ecology of chitin-degrading organisms.

- Distribution of chitinolytic organisms in space and time (Paper II, III)
  - Does the distribution of chitinolytic organisms follow the same general pattern as the combined bacterial community?
  - Is variation in chitinolytic activity accompanied by shifts in the respective chitinolytic community structure?

- Abundance of chitin degraders versus chitin consumers (Paper II, III, IV)
  - Is commensalism during chitin degradation a quantitatively important process in aquatic environments?

- Function-specific response to depletion of microbial diversity (Paper V)
  - Are widespread microbe-mediated functions less influenced by diversity in relation to more specific functions which are limited to a small subset of the total community?
Methods

Overview

Paper I is a literature review, bringing obtained results into context with previous research while revealing knowledge-gaps for further research. In Paper II the distribution of chitinase genes derived from the metagenomic Global Ocean Sampling dataset (Rusch et al. 2007) was analyzed in relation to the available metadata. Paper III is a field study assessing and trying to explain seasonal variation in chitinase gene assemblages in a lake during the course of a year. Paper IV and V describe experimental studies. In paper IV the fate of chitin hydrolysis products was studied after experimental addition of chitin to lake water. In Paper V the consequences of artificial manipulation of bacterial diversity on specific versus broad ecosystem functions was examined.

Isolation of chitinoclastic bacterial strains

Chitinoclastic freshwater bacterial strains were isolated to empirically validate the efficiency of established primers in amplifying bacterial chi-A genes. Freshly collected water from Lake Erken (50-100µl) was plated on an inorganic medium agar with added colloidal chitin as the only organic carbon and nitrogen source. The inorganic medium was based on WC medium (Guillard et al. 1972) with a few modifications containing the following compounds: 250 µmol L⁻¹CaCl₂•2H₂O, 150 µmol L⁻¹MgSO₄•7H₂O, 150 µmol L⁻¹NaHCO₃, 50 µmol L⁻¹K₂HPO₄•2H₂O, 1000 µmol L⁻¹NaNO₃, 11.7 µmol L⁻¹Na₂EDTA, 0.01 µmol L⁻¹CuSO₄•5H₂O, 0.08 µmol L⁻¹ZnSO₄•7H₂O, 0.04 µmol L⁻¹CoCl₂•6H₂O, 0.9 µmol L⁻¹MnCl₂•4H₂O, 0.03 µmol L⁻¹Na₂MoO₄•2H₂O, µmol L⁻¹388 H₃BO₃ and 35g L⁻¹ colloidal chitin (~95% H₂O). 13 isolates were phylogenetically characterized by 16S rRNA gene sequencing and used for establishing and testing PCR amplification of chitinase genes. The isolates were affiliated with a number of different bacterial taxonomic groups including Alpha-, Beta- and Gammaproteobacteria, CFB-group bacteria, Actinobacteria and Firmicutes (Table 2).
Table 2: List of chitinoclastic bacterial isolates and commercially available chitinolytic bacterial strains used for the setup of chitinase gene amplification. All isolates were grown on medium containing chitin as sole organic carbon and nitrogen source.

<table>
<thead>
<tr>
<th>strain ID</th>
<th>PCR amplification with primer</th>
<th>next cultured relative (% sequence similarity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-WC-06</td>
<td>+</td>
<td>Betaproteobacteria; Neisseriales; Neisseriaceae; (96%)</td>
</tr>
<tr>
<td>ER-WC-07a</td>
<td>+</td>
<td>Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces beijiangensis; (100%)</td>
</tr>
<tr>
<td>ER-WC-07b</td>
<td>+</td>
<td>Firmicutes; Bacillales; Bacillaceae; Bacillus; Bacillus cereus; (99%)</td>
</tr>
<tr>
<td>ER-WC-08*</td>
<td>+</td>
<td>Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Brevundimonas; (81%)</td>
</tr>
<tr>
<td>ER-WC-09</td>
<td>+</td>
<td>Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Caulobacter sp.; (99%)</td>
</tr>
<tr>
<td>ER-WC-11*</td>
<td>+</td>
<td>Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Janthinobacterium; (96%)</td>
</tr>
<tr>
<td>ER-WC-12</td>
<td>+</td>
<td>Gammaproteobacteria; Aeromonadales; Aeromonadaceae; Aeromonas sobria; (99%)</td>
</tr>
<tr>
<td>ER-WC-14</td>
<td>+</td>
<td>Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium; (92%)</td>
</tr>
<tr>
<td>ER-WC-15*</td>
<td>+</td>
<td>Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Flavobacterium sp.; (97%)</td>
</tr>
<tr>
<td>ER-WC-16*</td>
<td>+</td>
<td>Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Janthinobacterium; (95%)</td>
</tr>
<tr>
<td>ER-WC-17</td>
<td>+</td>
<td>Actinobacteria; Actinobacteridae; Actinomycetales; (97%)</td>
</tr>
<tr>
<td>ER-WC-18</td>
<td>+</td>
<td>Betaproteobacteria; Neisseriales; Neisseriaceae; Iodobacter; (97%)</td>
</tr>
<tr>
<td>ER-WC-21**</td>
<td></td>
<td>no pure culture</td>
</tr>
<tr>
<td>* sequenced bacterial strain possibly contaminated by other low abundant bacterial strains.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>** no clear 16S rRNA gene sequence obtained, mixed culture</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**PCR based molecular tools**

**Specific PCR amplification of chitinase genes**

Environmental DNA for PCR-based chitinase genes was used in Paper III and Paper V for T-RFLP (terminal restriction fragment lengths polymorphism) analysis and barcoded pyrosequencing, respectively. For the specific PCR amplification of chitinase genes, several previously published
primer pairs (Cottrell et al. 1999; Ramaiah et al. 2000; Williamson et al. 2000; LeCleir et al. 2004; Hobel et al. 2005; Xiao et al. 2005) were tested on 13 of the above mentioned bacterial isolates as well as on 3 commercially available chitinolytic strains (Table 2). All primer pairs were designed to amplify GH18 chitinase genes. The amplification with the primer pair ChiAF2/ChiAR2 (Hobel et al. 2005) was most successful, resulting in positive PCR reactions with amplicons of the expected size for 14 of the 16 bacterial test strains. For the two strains that did not amplify with the primer pair ChiAF2/ChiAR2, positive PCR reactions were obtained with another primer pair tested, indicating that all strains used for this validation were chitinolytic organisms containing GH18 chitinases. Furthermore, amplification products of the expected length were retrieved for all tested DNA extracts from environmental samples, even if also a number of unspecific bands were observed when PCR conditions were applied as published (Hobel et al. 2005).

*Chitinase* genes are characterized by high sequence variability. I therefore did not change the original PCR protocol to more stringent conditions in order to not exclude target DNA possibly containing mismatches from the PCR amplification. Amplicons of the expected length were instead excised from an agarose gel for further analysis by T-RFLP or 454 pyrosequencing.

PCR products for downstream applications that require modified primers, such as T-RFLP (attached fluorophor) or barcoded pyrosequencing (attached barcode and adaptor sequence), were obtained by applying a second PCR amplification. This was necessary since the modified primers did not result in an efficient and reproducible PCR reaction when applied to environmental samples. In the first step, original template DNA from environmental samples was amplified by the unmodified primer pair (30 cycles). Excised PCR products were then diluted and used as template for a second PCR using the respective modified primers (20 cycles).

**T-RFLP and construction of clone libraries**

T-RFLP is a fingerprint technique for overall comparison of community composition of previously PCR amplified gene products (Liu et al. 1997). This technique was applied in Paper V to assess the influence of experimental manipulations on bacterial communities and a functional guild. The combined bacterial community was targeted based on their 16S rRNA genes, and GH18 chitinase genes were used to describe changes in the chitinase-positive subset of the community.

T-RFLP fingerprinting only takes the most abundant community phylotypes into account, whereas less abundant populations are not detected. Natural bacterial communities usually have a highly skewed frequency distribution, with few abundant community members and a high number of rare phylotypes (Acinas et al. 2004; Pedros-Alio 2006). Hence this method is
not appropriate to assess diversity or phylotype richness in the typically very complex communities characteristic for most natural ecosystems.

To test the specificity of obtained PCR products, amplified *chitinase* gene fragments were cloned for sequencing (Paper V). PCR products were cloned into a vector, which was subsequently transformed into competent E. coil cells. The transgenic cells were individually grown on selective plates and approximately 30 clones were randomly selected from each library. The PCR-inserts in vectors were then sequenced following heat-lysis of individual clones and insert re-amplification.

**Barcoded Pyrosequencing**

Barcoded Pyrosequencing is a new high-throughput method for multiplexed sequencing of previously amplified PCR products from different samples (Meyer et al. 2008). The resulting sequence reads are separated according to origin and can subsequently be used for phylogenetic analyses or sorted into OTUs (operational taxonomic units) by a defined sequence similarity cutoff. In the latter case the resulting data can be used analogously to T-RFLP fingerprinting data. Due to the high sequencing depth made possible by the next-generation sequencing technology, data derived from barcoded pyrosequencing can also be used to estimate diversity or phylotype richness of bacterial communities in environmental samples.

**Rate measurements**

Uptake rates of glucose as well as the chitin hydrolysis products GlcNAc and (GlcNAc)$_2$ were measured by incubation of the sample water with the respective radiolabeled compound ($^3$H or $^{14}$C). After one hour of incubation, cells were collected on 0.2µm pore size membrane filters. The radioactivity on each filter was measured by liquid scintillation and reflects the amount of the respective compound incorporated in newly produced biomass. These uptake rates were measured as part of the study included in Paper IV to assess the importance of different chitin degradation intermediates as bacterial substrates.

Chitin and cellulose degradation rates were measured to probe the influence of an artificial manipulation of bacterial diversity on ecosystem functioning (Paper II, Paper V). These degradation rates were measured using the polymeric substrate analogs MUF-(GlcNAc)$_2$ and MUF-cellobioside where the released MUF fluoresce more intensely upon hydrolytic cleavage. Samples were incubated for approximately 8 hours and MUF-fluorescence, representing the amount of enzymatically processed substrate analogs, was measured by fluorescence spectroscopy.
Single cell analyses

Bacterial abundance, cell size and biomass

Total bacterial abundance was measured by first staining bacteria with a general DNA stain and subsequently counting bacteria manually by microscopy (Paper III and IV) or automatically by flow cytometry (Paper V).

Bacterial cell size and biomass was estimated by recording the length and widths of individual cells using microscopy followed by semi-automatic image analysis (Paper V).

MAR-CARD-FISH

MAR-CARD-FISH (microaudioradiography - catalyzed reporter deposition - fluorescence in situ hybridization) is a microscopy based technique to visualize bacteria that belong to a taxonomic target group and at the same time incorporate a specific substrate (Alonso et al. 2005). An environmental sample containing the indigenous bacteria is incubated with the radiolabeled test compound and subsequently fixed by paraformaldehyde and filtration. Due to the high sensitivity of this procedure, substrate concentrations close to environmental concentrations and short incubation times of less than one to a few hours are possible. Short incubation times lower the risk of cross-feeding between bacterial populations and hence prevent false-positive counts due to incorporation of secondary metabolites.

The fixed cells are first hybridized with a taxonomically specific oligonucleotide probe labeled with horseradish peroxidase (HRP). This probe binds to the ribosomal RNA of target cells and fluorophors get amplified and deposited at the site of the HRP activity. Silver grains then build up around radioactively labeled cells during microautoradiography processing. Hence it is possible to use a standard epifluorescence microscope to quantify the proportion of cells belonging to the taxonomic group under scrutiny and determine the portion of these cells incorporating the radiolabeled substrate.

CARD-MAR-FISH was applied in Paper IV to quantify bacterial cells incorporating chitin hydrolysis products in natural lake water samples and in experimental chitin amended cultures from the same lakes.

ELF®97-GlcNAc approach

Synthetic ELF®97-GlcNAc substrates can be used to identify surfaces (e.g. on cells or particles) with N-acetyl-glucosaminidase or chitinase activity (Baty et al. 2000). The water soluble and non-fluorescent ELF®97-GlcNAc serves as a substrate homolog for chitin and the water-insoluble and
intensely fluorescing ELF97-alcohol is cleaved off by enzyme hydrolysis. It is hence possible to quantify the portion of cells that express N-acetyl-
glucosaminidase or chitinase activity on their surface by fluorescence microscopy. ELF®97-GlcNAc was applied in Paper III to estimate the fraction of cells expressing chitinase activity and its seasonal fluctuation over a full year. In Paper IV the ELF®97-GlcNAc approach was used in combination with MAR and MAR-CARD-FISH to test the hypothesis that the proportion of chitinase active cells is far below the fraction of cells profiting from chitin derived hydrolysis products.

Sequence analysis and phylogeny
General sequence quality control and alignment
Based on e-values obtained via the Basic Local Alignment Search Tool BLAST (Altschul et al. 1997), *chitinase* genes were sorted out from metagenomic datasets (Paper II) or from unspecific PCR amplification products (Paper III).

For the alignment of amino acid sequences for downstream phylogenetic analysis, a seed alignment was created using full length amino acid sequences from available microbial genomes applying the homologs option in MAFFT v6.626 (Katoh et al. 2005). The alignment was then refined manually using the secondary structure of chitinase from *Serratia marcescens* (PDB entry 1E15) as a guide. All sequences were subsequently aligned to the seed alignment using the HMMer v2.3 software (Eddy 1998). In Paper II a Maximum Likelihood phylogenetic tree was constructed.

UniFrac distance
The UniFrac distance is calculated based on the branch length that connects two sequences in a phylogenetic tree (Lozupone et al. 2005). A distance matrix between sequences or phylotypes can be used for multivariate analyses. The UniFrac P-value indicates whether or not phylotypes from two different environments differ significantly in their phylogenetic composition. To generate P-values, phylotypes are randomly distributed within the phylogenetic tree. By repeating this procedure the null hypothesis that the shared branch lengths does not differ between the environments in the original and the randomized trees is tested. The UniFrac distance and UniFrac P-values were calculated in Paper II to hierarchically assess the influence of habitats defined by available metadata on the *chitinase* gene assemblages.
OTU based distance

As an alternative to the UniFrac distance, a distance matrix between sequences obtained from different environments can also be derived with distance measurements after first grouping available sequences based on a similarity cutoff value into OTUs. All OTUs are treated equally, regardless of their phylogenetic relationship to each other. This causes loss of phylogenetic information, but on the other hand it is an adequate method to analyze sequence data if little quality support is retrieved for phylogenetic analyses. This can for example happen as a result of short sequence length combined with high sequence variation.

In Paper III a Bray-Curtis distance matrix was generated after sorting chitinase protein sequences from pyrosequencing into OTUs based on complete linkage clustering with CD-HIT using 75% similarity cutoff.

Statistical data evaluation

Cluster analysis between the chitinase gene assemblages of different environments / samples was performed on a UniFrac distance matrix (Paper II) and on an OTU based distance matrix (Paper III).

To visualize if the metadata-based habitat definition in the metagenomic study of Paper II was also reflected in a grouping of the unique sampling sites, a principle components analysis (PCA) was performed.

ANOVA was performed to statistically evaluate whether or not there was variation of computed isoelectric points of chitinase fragments derived from different habitats (Paper II). A randomized ANOVA setup was used to account for the unbalanced data distribution.

In Paper V, the correlation between a dilution coefficient and measured functional parameters was statistically assessed using Spearman’s rank correlation. Correlations between distance matrices (T-RFLP-data: Bray-Curtis distance; functional parameters: Euclidean distance) were assessed using Mantel tests (Pearson’s correlation).
Results and Discussion

Distribution of chitinolytic organisms in space and time

To characterize decisive factors for the distribution and composition of chitinase genes over space and time, we pursued two different strategies. In Paper II we were using publicly available metagenome sequence data to assess the global distribution of aquatic chitinolytic organisms while coupling their distribution to environmental factors and bacterial communities in general. In Paper III we relied on PCR-based community assessment of chitinase genes to describe seasonal changes in the chitinolytic community in a temperate lake.

The global distribution of aquatic chitinase genes as a molecular marker for the functional guild of chitin degraders and 16S rRNA genes as a marker for the general bacterial community, were investigated as a function of habitat preference. We used DNA sequences from the Global Ocean Sampling (GOS) metagenome (Rusch et al. 2007) and the available metadata to define different habitats within categories (Table 3).

Table 3: Categories and subordinate habitats defined for the investigation of habitat based distribution patterns of chitinase and 16S rRNA genes.

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<thead>
<tr>
<th>category</th>
<th>habitat</th>
<th>abbreviation</th>
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<tr>
<td>salinity</td>
<td>freshwater</td>
<td>fre</td>
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<td></td>
<td>estuarine</td>
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<td></td>
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<td></td>
<td>hypersaline</td>
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<td>latitude</td>
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<tr>
<td></td>
<td>non-tropic</td>
<td>ntr</td>
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<tr>
<td>proximity of coast line</td>
<td>coastal</td>
<td>coa</td>
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<td></td>
<td>open ocean</td>
<td>ooc</td>
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<tr>
<td>geographic location</td>
<td>Caribbean Sea</td>
<td>car</td>
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<td></td>
<td>Galapagos Islands</td>
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<td></td>
<td>Indian Ocean</td>
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<td></td>
<td>North American East Coast</td>
<td>nor</td>
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<tr>
<td></td>
<td>Sargasso Sea</td>
<td>sar</td>
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We then tested the influence of habitat definitions for the different categories in explaining the composition of the investigated genes by hierarchical clustering according to the UniFrac phylogenetic distance of mixed categories (Figure 3).

**Figure 3. Hierarchical clustering of habitats derived from mixed categories based on the UniFrac phylogenetic distance of chitinase- and 16S rRNA genes.** Abbreviations were used as outlined in Table 3 and numbers in brackets indicate the number of included sequences. Numbers at the dendrogram nodes indicate jack-knife values. A: salinity / proximity of coast line; B salinity / latitude; C salinity / geographic location

Habitat definitions based on the category salinity, clearly had a major influence on the distribution of both chitinase and 16S rRNA gene pools. In general we found congruence between the distribution of chitinase genes and 16S rRNA genes, reflected by similar topography and branch lengths in the dendrograms for the categories salinity, latitude and proximity to coastline. This suggests that the major community drivers act on chitin-degrading organisms in the same way as they do on the total bacterial community (Figure 3).

The marked influence of salinity was corroborated by the observation that salinity affects the predicted isoelectric point of expressed chitinases (Figure 4). It is likely that salinity affects the combined proteome of bacteria in an analogous way, at least those proteins exposed to the external environment. This may provide an explanation for why salinity appears to influence the
composition of combined bacterial communities, regardless of their functional features.

Equally broad mechanisms might be responsible for the influence of other basic environmental parameters, such as temperature and pH, and account for analogous patterns in composition of chitinolytic organisms and combined bacterial communities.

Nevertheless, hierarchical clustering also suggests differences in the composition of chitinases and 16S rRNA genes among habitats based on the geographic location (Figure 3C). The distribution of chitin in the environment may affect the distribution of chitin degrading bacteria, as well as their relative contribution to the total bacterial community. Chitin supply is known to vary strongly between different geographic sites due to often temporally and locally restricted mass-occurrence of chitinous organisms such as crustacean zooplankton or diatoms. However, the GOS dataset that
was used in this study does not contain the necessary information to relate chitin degrading bacteria to the occurrence of sources of chitin.

Our further experiments build on the abovementioned results and previously observed variation in chitin degradation rates in freshwater in response to seasonal temperature and substrate influenced variations of chitin degradation rates (Warnes et al. 1982; Boyer 1994; Kirchman et al. 1999; LeCleir et al. 2006). By assessing the seasonal shifts in the phylogenetic composition of chitinase genes, we could indeed demonstrate major changes in the chitinase gene assemblage during the course of a year. A dendrogram based on Bray-Curtis distances reveals a principal separation into one cluster containing winter and spring samples and a second cluster containing summer and autumn samples (Figure 5). The Bray-Curtis distances between any pair of samples from the two main clusters was repeatedly close to 1, reflecting minimal overlap of chitinase OTUs between these two clusters.

Furthermore, a pronounced variation of OTU richness was observed during the course of a year, with richness minima often coinciding with major changes in community composition according to the cluster analyses (Figure 6 A). This observation implies a reoccurring collapse of the chitinolytic
community, followed by the establishment of a new quite different chitinolytic community.

The separation of the main clusters can be explained based on the abundance of crustacean zooplankton where samples in the summer/autumn–cluster reflect a community prevailing at high zooplankton biomass (Figure 6 B). Crustacean zooplankton is a major source of chitin in freshwater ecosystems. The chitin of these organisms is organized in the α-form which is different from the β-form chitin of diatoms that might be more important during other times of the year (e.g. winter and early spring). Such seasonal differences in the crystalline form of the available chitin may select for organisms specialized in utilization of the respective chitin source. It has indeed been shown, that a number of chitinolytic organisms prefer a single crystalline form of chitin (Ramaiah et al. 2000). Nonetheless, chitinolytic organisms are usually not obligate chitin degraders but can instead rely on a number of other substrates for metabolic use. Hence it is possible that chitin degraders specialize not only on a certain crystalline form of chitin, but on the whole suite of nutrients and organic compounds being released to the water during enhanced abundance of a certain organism group as e.g. crustacean zooplankton or diatoms.

Figure 6. (A) Dynamics of richness among the chitinase gene assemblage indicated by the chao1 estimator. (B) Dynamics of epilimnion phytoplankton (as indicated by chlorophyll a) and zooplankton (copepods and cladocerans) during the sampling period. For sample ss01 no data for zooplankton biomass are available. Plankton abundance was only assessed in epilimnic samples.
Abundance of chitin degraders versus chitin consumers

One of the aims of my thesis was to test if commensal metabolic use of chitin degradation products is an important trait in aquatic ecosystems. To address this we compared the occurrence of cells degrading chitin with those feeding on chitin degradation products.

Earlier attempts to quantify the frequency of chitin degrading bacteria by means of culturing range from 0.1-41% of the total bacteria (ZoBell et al. 1938; Ramaiah et al. 2000; Brzezinska et al. 2008). This huge variation might at least partly be explained by biases introduced by culturing approaches used in these early attempts to study bacterial chitin degradation. More recently, enzymes expressed in metagenomic libraries indicated that the frequency of chitin degraders in two estuarine and marine sampling sites ranged from 0.1-5.5% (Cottrell et al. 1999). Results from Paper II support this observation, while also extending these findings to 53 globally distributed aquatic sampling sites. Our analysis revealed that the frequency of chitinase genes in bacterial genomes vary from 0.2-4.5% in the free-living fraction (0.1-0.8µm) and from 0.3-5.8% in the particle fraction (> 0.8µm).

Planktonic bacterial cells expressing chitinase genes were quantified in Paper III and IV using the ELF®97-GlcNAc approach. The portion of chitinase-positive cells ranged from 0.0-1.9% in natural lake water. In chitin enriched lake water, up to 4.0% chitinase active cells could be detected. Simultaneous detection of chitinase positive cells and cells incorporating chitin hydrolysis products (Paper IV) revealed that the latter were in excess. Between 7 and 22% of the total community in untreated and chitin enriched lake water respectively incorporated GlcNAc and (GlcNAc)$_2$ in their biomass, compared to less than 1% of the cells being chitinase positive (Figure 7).

![Figure 7. Quantification of chitinase expressing cells (ELF®97-GlcNAc) and cells incorporating the chitin hydrolysis products GlcNAc and (GlcNAc)$_2$ as well as Glucose (Glc) as a reference substrate in Lake Erken and Lake Ekoln (untreated water and chitin enriched water).](image)

One word of caution is that chitinase activity estimates based on the ELF®97-GlcNAc approach may underestimate the active portion of the
community since not all types of chitinase genes might process this substrate homologue. However, the number of cells expressing chitinase genes (paper III) appears to be reasonable compared to the estimated frequency of chitinolytic cells based on metagenomic sequence data (paper II). Due to the pronounced difference in the number of chitinolytic cells and cells incorporating chitin hydrolysis products, Paper IV highlights the importance of commensal or ”cheater” strategies in aquatic chitin degradation. Similar mechanisms may also apply to other naturally produced biopolymers, implying that commensalism could be a generally important ecological strategy for bacteria in aquatic environments.

The identification of bacteria that incorporate chitin hydrolysis products revealed that the abundant and widespread (and so far uncultured) freshwater Actinobacteria belonging to the Ac1 cluster, profit the most from chitin enrichment. This was also the taxonomic group with the highest percentage of cells incorporating the chitin degradation products in biomass (Figure 8).

![Figure 8. Frequency of bacterial phyla that incorporate chitin hydrolysis products. A: GlcNAc, B: (GlcNAc)_2 in Lake Erken and Lake Ekoln in natural lake water and after chitin addition. Approximately 90 % of the Actinobacterial active cells belonged to the Ac1 group (not shown data).](image)

Freshwater Actinobacteria, with Ac1 bacteria as their most frequent representative, belong to the highly abundant freshwater phyla and can
contribute up to 70% of the cells in the bacterioplankton (Warnecke et al. 2005). So far it is not known to which ecological strategy they owe their success. Ac1 bacteria have been described as a group with a planktonic lifestyle. This was confirmed in Paper IV, where they were never observed attached to particles. In contrast, all other abundant non-Actinobacterial groups were frequently detected on particles. It would likely not be ecologically meaningful for organisms with a planktonic lifestyle to produce and maintain energetically expensive enzymes for particulate polymer degradation. In Paper IV, we therefore propose that members of the Ac1 group profit from commensal use of degradation intermediates produced by other hydrolysis-capable bacterial taxa.

Function-specific response to depletion of microbial diversity

Bacteria perform key ecosystem functions and have repeatedly been identified as important model organisms for diversity-functioning research (Hillebrand et al. 2001; Giller et al. 2004; Petchey et al. 2006). The role of biodiversity in determining the functioning of ecosystems is an important objective in ecology. For microbial communities, this has only recently been addressed experimentally and results are contradictory (Degens 1998; Mikola et al. 1999; Griffiths et al. 2000; Langenheder et al. 2005; Szabo et al. 2007). A possible explanation for such apparently contradictory results could be the existence of function specific responses. Broad-scale functions are characterized by high redundancy because basically all present species contribute to these functions. In essence, broad-scale functions would be less sensitive to species loss whereas more specific functions that are only carried out by a minority of the species present would be less redundant and could be more severely affected by species loss (Griffiths et al. 2000; Langenheder et al. 2006; Gamfeldt et al. 2008). However, also community composition and the presence of specific key species may be as important as species richness in determining ecosystem functioning (Covich et al. 2004; Downing 2005; Cardinale et al. 2006).

In Paper V a study of bacterial batch cultures hosting an experimentally created diversity gradient by dilution-to-extinction was performed to test the response of a number of different functional traits including broad-scale processes and more specific ones. Cultures were incubated with an inoculum size ranging from $10^5$ and $10^{-1}$ cells derived from natural lake water and for each dilution step, triplicates were prepared. The cultures were named in a way that sample names indicate the exponent for the inoculum size of the respective samples (e.g. 5 for $10^5$) and the letters (A-C) indicate the
triplicates for each inoculum size. Among the chosen measured functions, cell abundance, cell size, biomass and growth rate all represent broad-scale functions, whereas chitin degradation represents a specific function, apparently present in roughly 1% of the bacteria in aquatic environments. Frequency of bacteria with the ability to degrade cellulose, chosen as a the second specific function, is expected to be in the same order of magnitude, assuming that cellulose can be found in similar amounts as chitin in aquatic ecosystems (Gooday 1990a).

In contrast to our expectations, the results reveal stronger correlations between dilution gradient and broad-scale functions than between the dilution gradient and the specific functions, suggesting that diversity is more decisive for the broad-scale than for specific functions. However, Mantel-tests testing the relationship between functional and T-RFLP data also show that all measured functions, regardless of whether they are broad-scale or specific, were significantly related to the general bacterial community composition (Table 4).

Table 4: Summary of statistical comparisons of each measured function with dilution (Spearman’s rank correlations) and 16S rRNA gene community structure. Corresponding R, rs and P-values are shown. Mantel tests were performed on distance matrices of function and community composition as measured by T-RFLP on the 16S rRNA gene.

<table>
<thead>
<tr>
<th>function</th>
<th>Spearman’s rank correlation</th>
<th>Mantel test&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>rs</td>
<td>p-value&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>stationary phase abundance</td>
<td>0.88</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>maximum growth rate</td>
<td>-0.02</td>
<td>0.96</td>
</tr>
<tr>
<td>average cell volume</td>
<td>-0.89</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>biomass</td>
<td>0.88</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>chitin degradation rate (end)</td>
<td>0.29</td>
<td>0.39</td>
</tr>
<tr>
<td>cellulose degradation rate (end)</td>
<td>0.58</td>
<td>0.06</td>
</tr>
</tbody>
</table>

<sup>a</sup> 10 000 permutations  
<sup>b</sup> all significant p-values (< 0.05) were unaffected by multiple statistical testing

Chitin and cellulose degradation rates were only detectable in cultures with an inoculum size of at least $10^3$ cells (5A-3C) and in fact growth could only be detected in two cultures with an inoculum size smaller than $10^3$ cells (2B, 1C). Detectable chitinase and cellulose degradation rates coincided with cultures containing a specific phylotype, and this phylotype was enriched along the dilution series to make up nearly 100% in culture 3A (Figure 9). 16S rRNA gene sequence analyses identified this organism as an *Agrobacterium* sp. Even if the presence of this population in all cultures where chitinase and cellulose degradation was detected, this by no means prove that this organism is active in these processes, though this of course
seems plausible. Accordingly, this single population makes up nearly 100% of the community in culture 3A (Figure 9) and chitinase activity has previously been described for an *Agrobacterium* strain (DeAngelis et al. 2008).

![Figure 9: Heatmap illustrating the relative contribution of TRFs detected by T-RFLP of 16S rRNA genes with the following three restriction enzymes: haeII, hhaI and hinf. For each sample (column) the 3 restriction enzymes are plotted. The sample names indicate the exponent for the inoculum size of the respective samples (e.g. 5 for $10^5$) and the letters indicate the triplicate cultures for each inoculum size. In each row, the individual TRFs are plotted, which contribute between 0 (white) and 100% (black) of the total sample peak area. The increasingly darker values along the dilution gradient reflect the change in dominance from even communities towards communities dominated by single TRFs.](image)

Hence the low correlation between inoculum size and chitinase and cellulose degradation rates in our experiment appear to be caused by the presence of a single species. This population was apparently abundant in the inoculum and at the same time competitive enough to be detected in 7 out of altogether 9 analyzed cultures. Additionally, T-RFLP patterns indicated very low diversity, with a few abundant phylotypes present in cultures with an inoculum size of $10^4$ or less.

Based on the results in Paper V we claim that specific circumstances such as the identity of species within an inoculum and their ability to compete under the respective conditions are decisive for functional responses.
In spite of detecting chitinase degradation rates only in sample as 5A-3C, chitinase genes were detected also in the remaining two cultures 2B and 1C. (Figure 10).

Figure 10. Heatmap illustrating the relative contribution of TRFs detected by T-RFLP of chitinase genes with the following three restriction enzymes: hinfI, rsaI and mspl. For each sample (column) the 3 restriction enzymes are plotted. The sample names indicate the exponent for the inoculum size of the respective samples (e.g. 5 for $10^5$) and the letters indicate the triplicate cultures for each inoculum size. In each row, the individual TRFs are plotted, which contribute between 0 (white) and 81% (black) of the total sample peak area.

This can be explained by non-functional gene homologues or caused by suppression of chitinase gene expression in cultures 2B and 1C. However, T-RFLP patterns of chitinase genes suggest the presence of 2-4 different chitinase phylotypes that were detected in the cultures 2B/1C, but also in most of the other remaining cultures. This observation supports that at least one of the detected chitinase genes in culture 2B or 1C would also code for an active protein.
Conclusions and future perspectives

In brief, the following conclusions can be drawn from Papers II-V:

1. The distribution of chitinolytic organisms was in most of the examined cases congruent to that of the total bacterial community. Among the tested factors, salinity had a superior influence on the gene distribution. I also show that salinity affects the isoelectric point and therefore the general protein architecture of chitinase genes. Such salinity induced shifts possibly shape the combined proteome of aquatic bacteria. It may therefore explain the typically very strong effect of salinity on overall bacterial community composition, regardless of their functional affiliation. Only if grouped based on geographic locations, distribution patterns of chitinolytic organisms disagreed with those of the total bacterial community. Chitin supply can vary dramatically between geographic sites due to temporally and locally restricted mass development of chitinous organisms such as crustacean zooplankton or diatoms. I therefore suggest that chitin availability shapes the chitinolytic community.

2. A seasonal survey confirmed the abovementioned assumptions, showing that seasonal shifts in zooplankton biomass were accompanied by changes in the chitinolytic community composition. These results further suggest that chitinolytic communities are highly dynamic with minimal species overlap between different periods of the year.

3. Experiments based on single cell methods show that commensal consumption of chitin hydrolysis products is a quantitatively important substrate acquisition strategy in freshwater environments. Our results also suggest that Ac1-group *Actinobacteria*, one of the most abundant freshwater bacterial groups, profit from exactly this ecophysiological strategy.

4. Experiments based on artificially created gradients in bacterial diversity demonstrated the importance of environmental conditions – in our case the presence of a key species - to the specific functional response. The influence of the overall bacterial diversity on the other hand was subordinate in the respective treatments.
It was stated in the introduction of this thesis that chitin does not accumulate in nature. Such observation entails that chitin degradation rates ultimately depend on chitin production rates rather than on other environmental state variables. This leads to the inevitable question of why then it is important to examine and understand the detailed mechanisms and ecology of organisms responsible for chitin degradation?

One answer to this question is that changes in environmental parameters can lead to shifts in the chitin utilizing community and/or alter mechanisms of chitin utilization. This in turn will influence the fate and role of this biopolymer in nature; e.g. the ratio of rapidly mineralized chitin to newly assimilated degradation products. Due to the enormous global production and degradation of chitin, this may influence the mass balances of carbon as well as nitrogen cycles in the biosphere. Hence in the future research it would be important to put more emphasis on the downstream pathways, mechanisms and biological effects of degraded chitin rather than simply focusing on degradation rates.

In anthropogenic engineered systems, such as waste water treatment plants or more specifically the chitinous waste from the seafood industry, other factors than chitin supply might regulate the efficiency and rate of polymer degradation. To obtain stability and predictability in such systems, detailed knowledge about process regulation will be advantageous. Another pertinent question is why it would be essential to gain knowledge about the community responsible for chitin use, if degradation and mineralization rates can be predicted from more basic physical and chemical driver variables? However, the inconsistent influence of temperature on chitin degradation rates (Paper I) contradicts the idea of simple first-order predictability for this complex biological degradation process. Furthermore, data in this thesis provide evidence that chitin degradation may largely depend on the presence of a key-species rather than general diversity indices. This points to the importance of all the environmental conditions that are conducive for such key species, in controlling chitin degradation (Paper V).

In the contemporary situation, where natural mass fluxes of organic matter are increasingly influenced by anthropogenic activities, it will be important to reliably and precisely predict mass fluxes in engineered and natural systems, locally restricted as well as on a global scale. For this purpose, we will need to understand and classify how general features of a functional community as e.g. species diversity, key-players or species interactions as commensalisms interrelate to the effectiveness and stability of a certain process. Since I could not find any influence of dispersal on the global distribution of chitin degraders, it remains to find out if similar environmental circumstances, such as mass development of zooplankton, will favor the development of the same key species in the respective environments. It is furthermore conceivable, that by a permanent and effective removal of hydrolyzed chitin mediates by commensals, expression
and therefore activity of chitinases will be enhanced. A further aspect that in my opinion should (and likely will) attract attention in future research is the mapping of changes in activity patterns due to altered gene expression versus community shifts on the respective relevant time scales.

This thesis specifically focuses on the process of chitin degradation and the associated bacterial community. Chitin was chosen partly because its degradation is easier to target than degradation of other polymers such as the structurally heterogeneous lignin and humic acids or even cellulose. This is at least partly due to its quite simple molecular structure and the existence of primer-systems targeting conserved parts of the genes coding for the chitin manipulating machinery. Chitin further holds a key role in C and N cycling due to its enormous global abundance. Chitin degradation might be used as a general model for understanding mechanisms involved in microbial degradation of polymeric organic substances in nature.
Swedish Summary (Sammanfattning)

Kitin är en av de kvantitativt mest betydelsefulla organiska föreningarna i biosfären, där de spelar en viktig roll som struktuväv i kräftdjur, insekter, svampar och vissa växtplankton. Enbart i akvatiska ekosystem uppgår den globala produktionen av kitin till $10^{10-11}$ ton och avsaknaden av betydande ackumulering i naturen indikerar förekomsten av en effektiv nedbrytning och omsättningen av denna biopolymer spelar därmed en central roll i globala kretslopp av både kväve som kol. I akvatiska system svarar bakterier för den huvudsakliga nedbrytningen av kitin, och det övergripande målet med denna avhandling var att öka vår förståelse av denna process och förbättra vår kunskap om dessa kitin-nedbrytarens funktion, mångfald och ekologi i akvatiska ekosystem. En inledande litteraturstudie som ingår i denna avhandling (artikel 1) identifierade till exempel flera områden där vår kunskap om kitin-nedbrytning och de organismer som utför denna process är mycket bristfällig och ofullständig. Exempel på sådana kunskapsluckor är information om i vilken grad kitin-nedbrytarnas mångfald och artsammansättning påverkar kitin-nedbrytning och vilka miljöfaktorer som är betydelsefulla i kontrollen av denna grupp av mikroorganismer. Med utgångspunkt delvis från denna litteraturöversikt genomfördes en rad fältstudier och experiment där följande slutsatser kan dras (artikel 2-5):

1. En global jämförelse av kitinaser i akvatiska metagenomiska dataset visade att kitin-nedbrytande organismers artsammansättning och samhällsstuktur till stora delar sammanföll med variation i det totala bakteriesamhällets artsammansättning. Bland en rad studerade miljöfaktorer visade sig salthalten ha en primär inverkan på båda dessa typer av bakteriesamhällen. Salthalten påverkade även de uttryckta proteinernas karaktär och laddning vilket skulle kunna förklara hur en faktor som salthalt kan påverka hela det bakteriella sammanhållets sammansättningen oberoende av deras biogeokemiska funktion och roll i ekosystemet. Endast i de fall då bakteriesamhällets artsammansättning analyseras mot bakgrund av specifika geografiska platser eller områden skilde sig kitin-nedbrytarnas fördelning från det kombinerade/totala bakteriesamhället. Dessa skillnader härrör förmodligen från lokala skillnader i kitinsubstratens tillgänglighet och form, då detta kan variera kraftigt mellan olika geografiska områden, till stor del beroende på spatiellt aggererad massförekomst av
planktoniska organismer som innehåller kitin; e.g. kräftdjur zooplankton eller kiselalger. Med bakgrund av dessa observationer föreslås att tillgänglighet på denna potentiella födresurs är en faktor har stor betydelse för kontrollen och aktivitet inom det kitin-nedbrytande bakteriesamhället.

2. För att vidare studera hur övrig biota och andra potentiella miljövariabler såsom kitin-tillgänglighet påverkade det kitin-nedbrytande samhällets mångfald, sammansättning och funktion i avsaknad av salinitets-kontroll, genomfördes en utförlig säsongsstudie i en välstuderad dimiktisk sjö. Genom att utnyttja nya kraftfulla sekvenseringsmetoder kunde samhällets sammansättning och mångfald följas och jämföras över tid. Resultat från studien visar tydligt på en dramatisk variation i det kitinolytiska samhällets sammansättning över årets säsonger, där mycket få kitin-nedbrytare förekommer i sjön under några längre tidsperioder. Genom att jämföra dessa samhällsdata med ekosystemets karaktär framgår det tydligt att förändringar i djurplankton-samhällets biomassa och andra större förändringar i ekosystemets struktur och funktion (e.g. skiktning och ombländning, växtplankton-blooming) åtföljs av markanta förändringar i det kitin-nedbrytande samhällets sammansättning. Detta tolkades som en stark resurs-kontroll av det kitin-nedbrytande bakteriesamhället.

3. I kontrollerade experiment som baserades på kitin-anrikade inkubationer med sjövatten, utnyttjades enkelt-cells metoder för att studera hur olika bakteriesläkten uttryckte kitinas-aktivitet och utnyttjade de nedbrytningsprodukter som genererades av dem enzymatiska aktivitet. Dessa studier visade tydligt att en stor del av de bakterier som uppenbart drar nytta av nedbrytningsprodukter från kitin-nedbrytningen, inte själva är kapabla att bryta ner kitin. Istället förlitar de sig på andra bakteriegruppers hydrolytiska aktivitet, en ”kommensalistisk” eller ”fuskande” livsstil som skulle kunna vara mycket viktig i akvatiska miljöer. En indikation på dess potentiella betydelse är det faktum att den ofta kvantitativt dominanta sötvattensbakterie-gruppen *Actinobacteria* Acl verkar utnyttja just denna metabola strategi. Med bakgrund av detta föreslår vi att detta kan vara en viktig ekologisk strategi även för nedbrytning av andra polymerer och i andra akvatiska miljöer.

4. Slutligen visade sjövattenexperiment som bygger på artificiellt skapade graderier i bakteriell mångfald och artrikedom på betydelsen av specifika nyckel-populationer snarare än mångfald *sensu* det totala antalet arter för vidmakthållandet av mer specifika biogeokemiska processer såsom nedbrytning av kitin och andra biopolymerer. Detta står i kontrast till mer generella samhälls-funktioner där artrikedom är
positivt kopplat till grundläggande samhällsfunktioner som tex bakterietillväxt.

Kitin är en av de vanligaste organiska föreningarna i biosfären och en ökad kunskap om dess förekomst och ekologin hos de organismer som utför denna process kommer sannolikt att förbättra våra möjligheter att förutse hur mikrobiella ekosystemtjänster kan förändras i skenet av pågående och framtida miljöförändringar. Nedbrytning av kitin är även en process som av många olika anledningar är en lämplig modell för att studera mikrobiell omsättning av organiskt material i biosfären och kan också bidra till en ökad insikt om kopplingarna mellan biogeokemiska processer och de ytterst komplexa mikrobiella samhällen som återfinns överallt i naturen.

Das Ziel meiner Arbeit war es Abbauprozesse zu untersuchen, die notwendig sind, um Chitin in seine anorganischen oder biologisch andersweitig verfügbaren Grundbausteine zu zerlegen. Besondere Aufmerksamkeit habe ich dabei auf das Vorkommen, die Artenzusammensetzung und Ökologie der Organismen gerichtet, welche die notwendige enzymatische Austattung haben, um Chitin abzubauen: das sind in Gewässer-Ökosystemen vor allem Bakterien. Meine Doktorarbeit enthält vier experimentelle Studien (Artikel 2-4), deren Ergebnisse hier kurz zusammengefasst werden sollen:


4. In einer vierten Studie wurden Experimente durchgeführt, um den Einfluss eines künstlich dezimierten bakteriellen Artenreichtums auf


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