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The Niches of Bacterial Populations in Productive Waters

Examples from Coastal Waters and Four Eutrophic Lakes

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Abstract
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Recent research in microbial ecology has focused on how aquatic bacterial communities are assembled. Only a few of these studies follow a "Gleasonian" approach where the roles of single bacterial populations are in focus. In this thesis, novel molecular tools were used to describe the distribution and evolutionary relationships of microbes in productive aquatic environments. Many new phylogenetic groups of bacteria were identified, likely representing bacterial populations restricted to productive freshwaters. I also addressed the dynamics and functional role of individual bacterial populations in eutrophic lakes and brackish environments with a focus on either biogeochemically significant or potentially pathogenic representatives. *Flavobacteria* blooms were observed, on occasions characterized by high heterotrophic production. In addition to high temporal dynamics microbial community composition and function differed on the spatial scale, as exemplified by free-living and *Cyanobacteria*-associated habitats. At the community scale, microbial processes, such as biomass production and substrate uptake could be predicted from the presence and absence of individual bacterial populations. I also studied the niches of potentially pathogenic *Vibrio* populations in various coastal waters. Using a novel culture-independent method, a *V. cholerae* population was detected along the entire Swedish coastline. Results from an environmental survey and a laboratory mesocosm experiment reveal that phytoplankton-derived dissolved organic matter enhance the growth of *V. cholerae* and other *Vibrio* spp. and hence create a largely overlooked niche for these heterotrophic bacteria. This thesis and future work on the role of individual bacterial populations will facilitate predictions of biogeochemical cycles and the distribution of bacteria in the context of global climate change and local eutrophication.

Keywords: diversity, 16S rRNA, phytoplankton, bloom, pathogen, carbon cycle

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To the memory of my father

"Laughter can free your soul"

List of papers

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

I Eiler, A., and S. Bertilsson. 2004. Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environ. Microbiol 6: 1228–1243*.

II Eiler, A., and S. Bertilsson. Blooms of *Flavobacteria* in four productive lakes. *Submitted*.

III Bertilsson, S., Eiler, A., Nordqvist, A., and N.O.G. Jørgensen. Links between bacterial production, amino acid utilization and community composition in productive lakes. *Manuscript*.

IV Eiler, A., Olsson, J.A., and S. Bertilsson. 2006. Diurnal variations in the auto- and heterotrophic activity of cyanobacterial phycospheres (*Gloeotrichia echinulata*) and the identity of attached bacteria. *Freshwater Biol. 51: 298–311*.

V Eiler, A., Johansson, M., and S. Bertilsson. 2006. Environmental influences on *Vibrio* populations in northern temperate and boreal coastal waters (Baltic and Skagerrak Seas). *Appl. Environ. Microbiol. 72: 6004-6011.*

VI Eiler, A., Gonzalez-Rey, C., Allen, S., and S. Bertilsson. Growth response of *Vibrio cholerae* and other *Vibrio* spp. to cyanobacterial dissolved organic matter and temperature in brackish water. *Submitted*.

Contents

Abbreviations

CCA	Canonical-Correspondence-Analysis
CFU	Colony-Forming Units
chl-*a*	Chlorophyll-*a*
DFAA	Dissolved Free Amino Acids
DGGE	Denaturant Gradient Gel Electrophoresis
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
HR-T-RFLP	High-Resolution-Terminal-Restriction-Fragment-Length-Polymorphism
nMDS	nonmetric Multi Dimensional Scaling
OTU	Operational Taxonomic Unit
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PLS	Partial Least Square projection to latent structures
qc-PCR	Quantitative-competitive Polymerase Chain Reaction
SSU rRNA	Small Subunit of the ribosomal RNA
T-RF	Terminal-Restriction-Fragment
T-RFLP	Terminal-Restriction-Fragment-Length-Polymorphism
VBNC	Viable But Non-Culturable
16S rDNA	Gene coding for the RNA in the small (16S) subunit of the bacterial ribosome
16S rRNA	RNA in the small (16S) subunit of the bacterial ribosome

INTRODUCTION

Bacteria represent a major constituent of the living biomass in aquatic environments (179). These organisms facilitate and regulate biochemical cycles of the major elements in marine and fresh waters, like C, N, P, S, and Fe (e.g. 20 and 106). They are major food sources for higher trophic levels within the aquatic food chain (132), and many globally significant bacterial pathogens originate from aquatic environments (26, 151). To fully understand biogeochemical fluxes and manage hazards related to water-borne disease, we need to study the specific roles of different microorganisms in their environment. However, our understanding of the ecology, physiology and distribution of bacteria in natural environments is incomplete. Most current knowledge originates from a mere 10,000 cultured isolates and only a few of these are studied extensively. Another problem is that these isolates rarely represent populations that are abundant in natural aquatic habitats (61).

Molecular tools have greatly increased our understanding of the diverse microbial world. Phylogenetic marker genes, like the 16S rRNA gene, allow us to study microbial diversity in natural environments at different levels of taxonomic resolution. Operational taxonomic units (OTUs) are defined based on features such as a certain degree of 16S rRNA sequence similarity, gene length heterogeneity or location of sequence-specific restriction sites. We know now that less than a liter of water can harbor more than 100 OTUs (Table 1), which may correspond to more than a thousand bacterial "species" (142, 166) and to even more bacterial ecotypes (44, 141, 166).

Despite the current controversy about the bacterial species concept, recent studies have adapted general ecological theories to the microbial world. A few examples are: the island biogeography theory and species-area relationship (54, 71, 103, 137), the metacommunity concept (30, 98, 102), the niche concept (44, 141, 178), interspecific interactions (competition, predation etc.; see for example 96, 127, 152, 153), the redundancy theory (145, 161), etc.

Based on these and other studies, assembly rules for aquatic bacterial communities can be summarized in a conceptual figure (Fig. 1). For example, the metacommunity concept emphasizes the impor-

tance of dispersal that links local habitats to a metacommunity (59, 180). It is based on the island biogeography theory by MacArthur and Wilson (109) that emphasizes the importance of migration between islands (dispersal within a metacommunity) as well as fragmentation and distribution of habitats (islands) regulating the distribution of organisms. One observation based on the island biography theory is the species–area relationship, which states that larger islands encounter more diverse communities (e.g. continents). MacArthur and Wilson (109) also stated that the species-area relationship is strongly influenced by dispersal (immigration) and habitat distribution.

Habitat type	Habitat	References	Diversity estimates	
			Chao-1	Reciprocal Simpson´s index
marine	*Areachon bay, French Atlantic coast*	Benlloch et al. 1995	112	32.3
	Prévost lagoon, French Mediterranean coast	Benlloch et al. 1995	144	167
	Long Island coast, USA	Kelly and Chistoserdov 2001	75	32.3
	Columbia River estuary	Crump et al. 1999	205	490
freshwater	*Lake Cadagno, Switzerland*	Bosshard et al. 2000	138	29.4
	Lake Ekoln, Sweden	**paper I**	118	1.8
	Lake Erken, Sweden	**paper I**	92	5.8
	Lake Limmaren, Sweden	**paper I**	154	12.4
	Lake Vallentunasjön, Sweden	**paper I**	98	3.6
	Mono Lake, CA, USA	Humayoun et al. 2003	112	18.6
	Bugach, Siberian reservoir, Russia	Trusova and Gladyshev 2002	85	26.3
	Lesnoi, Siberian reservoir, Russia	Trusova and Gladyshev 2002	33	16.1
	Lake Blankaart, The Netherlands	Van der Gucht et al. 2005	72	34.8
	Lake Visvijver, The Netherlands	Van der Gucht et al. 2005	108	47.8
	Lake Maten 12, The Netherlands	Van der Gucht et al. 2005	166	115.0
	Lake Maten 13, The Netherlands	Van der Gucht et al. 2005	191	93.4
	Lake Pavin, France	Boucher et al. 2005	49	40.0
	Sep reservoir, France	Boucher et al. 2005	42	27.0
	Lake Stechlin, Germany	Allgaier et al. unpublished	130	6.1
	Lake Breiter Luzin, Germany	Allgaier et al. unpublished	30	13.5
	Lake Tiefwaren, Germany	Allgaier et al. unpublished	202	42.9
	Lake Grosse Fuchskuhle, Germany	Allgaier et al. unpublished	42	19.0

Table 1. Summary of chao-1 estimates (22) and reciprocal Simpson´s index (66) retrieved from 16S rDNA-based clone libraries.

Additional studies have investigated influences of resources on bacterial communities. Resource quality (29, 90, 171) and quantity (34) seem to alter bacterial community composition. For instance, dissolved organic matter (DOM) originating either from allochthonous or autochthonous DOM sources results in different degrading communities (29, 60, 90, 119).

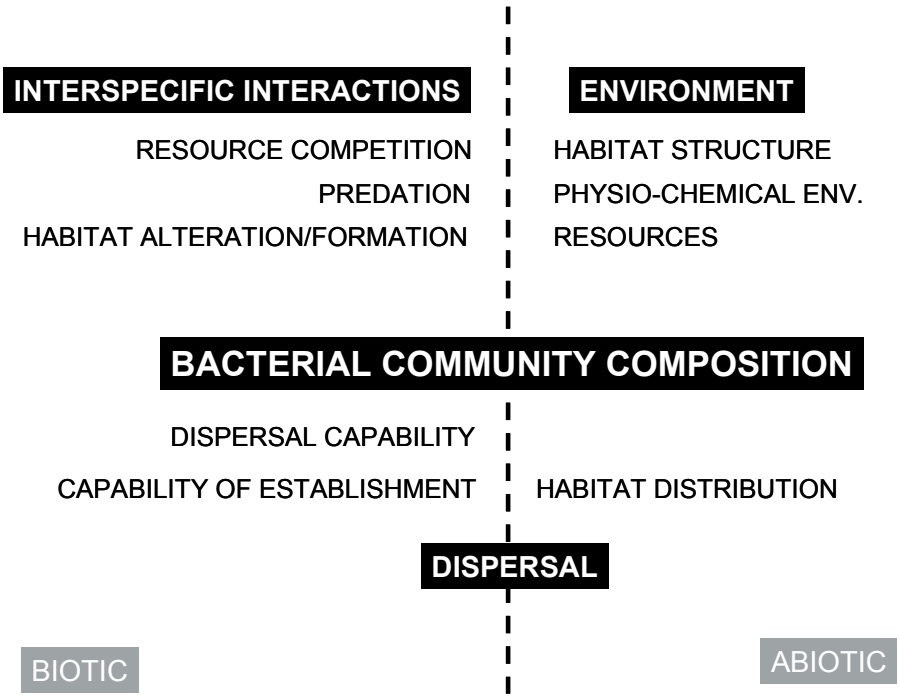


Figure 1. Schematic example of biotic and abiotic forces shaping bacterial communities. The relative importance of the different forces, like predation, resources, dispersal on microbial communities is poorly understood.

Although, there seem to be rules for community assembly of aquatic microbes, to use a "Gleasonian" approach (50) can often be an advantage; i.e. to focus on distinct bacterial populations that can be reliably identified and quantified in aquatic environments rather than on entire microbial community structure and diversity. Bacterial communities can be separated hierarchically and grouped into taxonomic units (i.e. phylum, class, genus) or functional groups. In this thesis, 16S rRNA based methods were used to study taxonomic groups, e.g. dominant bacterial community members, class *Flavobacteria*, genus *Vibrio* and subgroups therein. When studying the ecology of single populations, the niche concept can be useful (37, 76). A niche is defined by the environmental limits within an organism can survive, grow, reproduce and maintain a viable population. Numerous studies have reported the apparent niche partitioning between freshwater and marine bacteria (51, 52, 112, 185). Comparative analyses of 16S rRNA gene sequences have revealed globally distributed freshwater lineages phylogenetically distinct from marine lineages.

There are several more examples of physico-chemical growth constraints of microorganisms. Lindström and colleagues (101) studied the apparent niches of ubiquitous freshwater bacterial lineages along a gradient of 15 lakes. Different bacterial lineages (populations) seemed to occur more frequently at different ranges of pH, lake retention time and temperature. For example, a subpopulation of the ubiquitous *Polynucleobacter* lineage occurred only in habitats with low pH. In other studies, UV-radiation and allochthonous sources of DOM were identified as regulating factors of an actinobacterial lineage (18, 174).

Niche dimensions are not only limited to physico-chemical constraints, but also include the resources that an organism requires (Fig. 1). For example, *Flavobacteria* are described as chemoorganotrophs that thrive on biopolymers such as cellulose, chitin and pectin for growth (11) and proliferate in DOM-rich environments (91). In contrast, bacteria affiliated with the acI lineage of the *Actinobacteria* seem to be more abundant in environments with allochthonous material as the main DOM source (18, 52, 100). Laboratory studies with cultured isolates have provided some preliminary information on the fundamental niches of some bacterial populations, including the acceptable tolerance limits (e.g. salinity and temperature) and critical nutrients. Whether a population can occur and persist in a certain niche in nature depends on two further factors: dispersal and interspecific interactions (Fig. 1). Dispersal depends on the ability of populations to colonize a certain environment and the mechanisms and processes leading to a transfer of organisms between habitats (e.g. water flow; see reference 102). Competition for resources and predation by other populations may further affect the potential to maintain viable populations in a certain habitat. Also grazing (for example see reference 96) and viral infection (150) are involved in shaping bacterial community composition. Interspecific interactions can be more cryptic. For example phytoplankton blooms can alter the physico-chemical environment (e.g. redox potential and nutrient availability), as well as predation pressure. To conclude, the regulation of bacterial populations is an interplay between dispersal, access to resources, physico-chemical growth constraints, and mortality factors.

Phytoplankton blooms are potential model systems to study the effects of these five factors. These episodes of elevated cyanobacterial/algal population abundance are common in productive coastal and freshwater systems. These episodic events can have a strong effect on the habitat by changing the chemical environment and habitat structure. Direct attachment of bacterial cells to phytoplankton blooms provides new niches for biofilm-forming bacteria. The inter-

specific interactions of the phytoplankton with the closely associated bacterial populations can range from resource competition to mutualism.
The present thesis assess the role of phytoplankton blooms and other environmental variables in controlling and modulating bacterial community assemblies and the role of such conditions in creating niches for specific bacterial populations. The environmental relevance of the work relates to an increased understanding of how biogeochemical cycles are regulated, of the persistence of phytoplankton blooms, and of the ecology as well as distribution patterns of potentially pathogenic bacteria in productive aquatic environments.

Phytoplankton blooms

Episodes of markedly enhanced phytoplankton biomass compared to the yearly average, estimated as concentrations of cells or chlorophyll-a (chl-*a*), are referred to as blooms. Phytoplankton blooms are usually comprised of only one or two species. These are often species that can actively migrate through the water-column using gas-vacuoles or flagella (120).
Massive developments of microeukaryote *Algae* and *Cyanobacteria* are common in costal regions of the world oceans, freshwaters and hypersaline environments. High abundances of these phytoplanktons can occur when conditions and resources are suitable for the various species. To a large extent, cell abundances, species composition, vertical distribution, longevity and timing of the blooms, can be explained by climatic and meterological conditions which influence light and nutrient availability as well as the degree of stratification (120).
Phytoplankton blooms can have a marked economic and social impact. Many species release potent toxins with health problems and fish-kills as consequences. Enzymatic degradation by heterotrophic bacteria is one mechanism for toxin removal, paralleled by solar driven photochemical degradation and adsorption to particles followed by sediment burial (156). Phytoplankton blooms may also impair water treatment processes by causing filter blockage and hence increase the costs and challenges involved in purification while also causing significant taste and odor problems (33, 94). An additional consequence of phytoplankton blooms and their interactions with aquatic heterotrophic bacteria is the deoxygenation of the water column during degradation of phytoplankton biomass at the collapse of blooms. This can lead to fish-kills and redox-driven release of nutrients from the sediments. Another largely overlooked health hazard

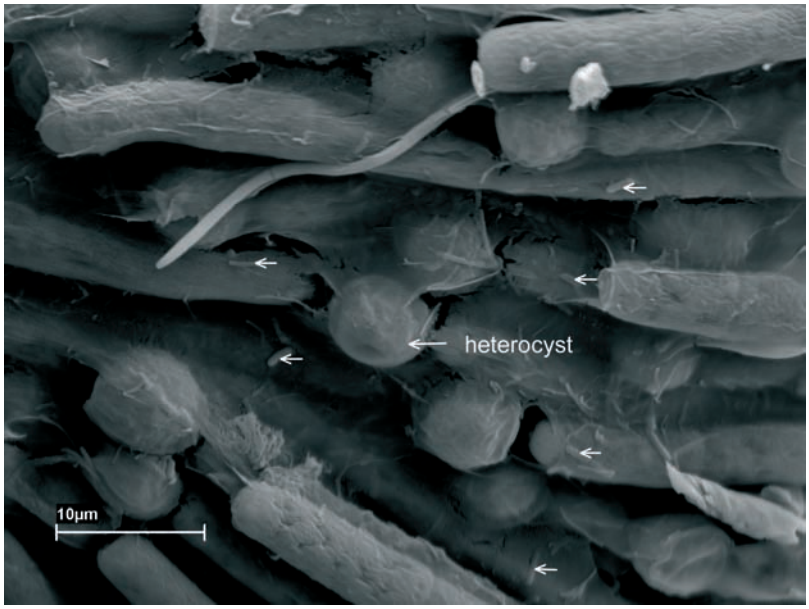
that may result from phytoplankton blooms is the possible promotion of various pathogenic microorganisms that are able to exploit the high amounts of carbon substrates released and in other ways benefit from the conditions created by the bloom (40, 122).

Biological effects of phytoplankton blooms

Phytoplankton blooms can have dramatic effects on the aquatic biota by radically changing the habitat structure. Their photosynthetic activity can increase pH and redox potential in the water column. They affect light conditions, and their activities and growth also change the availability of nutrients. As a consequence of nitrogen fixation carried out by different *Cyanobacteria*, systems can shift from a nitrogen to a phosphorus-limited state (149). Even if *Algae* and *Cyanobacteria* compete with other microorganisms for nutrients, their vertical migration can lead to a net import of nitrogen, phosphorous and other micronutrients to the nutrient depleted photic zones of stratified aquatic environments.

As primary producers, *Algae* and *Cyanobacteria* are important sources of organic matter in most aquatic systems. Fractions of their photosynthetic products are released as particulate or dissolved organic matter during cell lysis due to viral infection, grazing, or other mortality factors (13). A variable fraction of the fixed carbon is also released as DOM from actively growing cells. Both environmental conditions and the composition of the phytoplankton assemblage influence the composition of the algal-derived DOM (64, 79, 163) and the major inputs are typically associated with periods of intense growth of phytoplankton (39). Although little is known about the exact chemical composition of phytoplankton-derived dissolved organic matter, previous studies have shown that exudates like carbohydrates, organic acids, and dissolved combined and free amino acids (DFAA) can be significant factors in the regulation of associated heterotrophic bacteria (45, 183). The surges of DOM released by phytoplankton create temporally and spatially constrained new niches that are rapidly filled by heterotrophic microorganisms (12, 13). An inference of this is that heterotrophic bacterial growth (34, 159), bacterial abundance (38, 92) and ectoenzyme activity (23, 24) increase substantially during periods of enhanced phytoplankton activity and abundance.

A



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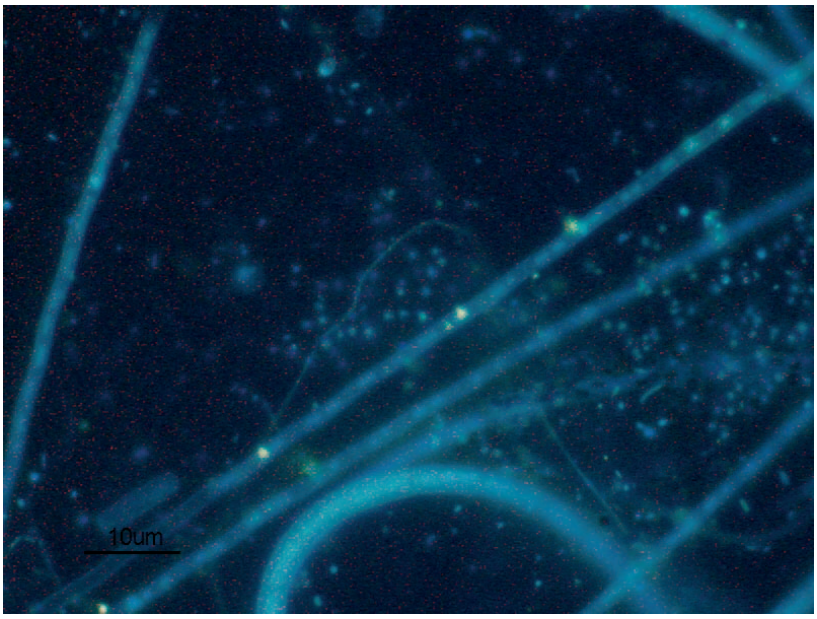

Figure 2. Cyanobacterial colonies extensively colonized by bacteria. (A) Scanning electron micrographs of *Gloeotrichia echinulata* colonies demonstrating the presence of individual bacterial cells embedded in the mucus surrounding *G. echinulata* filaments. Small arrows indicate non-cyanobacterial cells. (B) Epifluorescence micrographs of cyanobacterial filaments and loosely associated bacteria.

Furthermore, *Algae* and *Cyanobacteria* can be intensively colonized by bacteria (8, 14, 16, 155, 182; see also Fig. 2). Bacterial colonization of phytoplankton has for example been observed on *Aphanizomenon* heterocysts (124), *Nodularia spumigena* filaments (144), *Trichodesmium* filaments (118, 125), *Alexandrium tamarense* (155) and various diatoms (14, 55). Attachment to phytoplankton may provide a refuge from grazing and may also change the dispersal and physiology of certain bacterial populations. Attached bacteria are on the one hand usually less abundant than free-living bacteria in the water column, but on the other hand, they are believed to represent a metabolically more active fraction of the community (155). Since spatially constrained patches of organic compounds are created around these assemblages, aquatic phycospheres are commonly recognized as hotspots of microbial activity (182).

Attachment may for example promote certain fast-growing heterotrophic bacteria that are able to cope with the frequent (diurnal) alterations in redox potential created by concurrent respiration and photosynthetic oxygen production. Such conditions could for example favor microaerophilic bacterial pathogens that are adapted to alternate between aquatic systems and the intestinal environment.

The factors outlined above suggest that enhanced phytoplankton biomass is likely to structure and control the associated bacterial community. Some empirical support exists, e.g. the use of 16S rRNA-based fingerprinting methods have revealed shifts in bacterial community composition during the course of phytoplankton blooms (2, 41, 80, 93, 130). Dramatic changes in bacterial community composition have also been observed upon viral-induced termination of a cyanobacterial bloom (171). More direct evidence that cyanobacterial blooms favor certain heterotrophic bacteria is provided in a study showing that the abundance of a *Cytophaga* species capable of lysing cyanobacterial cells was tightly coupled to the development and decline of a freshwater bloom event (136).

Typical freshwater bacteria: an overview

In lakes, three phyla, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*, usually dominate the bacterioplankton communities (51, 52, 112, 185). Other phyla that seems to be abundant in freshwaters are: *Firmicutes, Acidobacteria, Verrucomicrobia, Planctomycetes, Nitrospira* and several candidate divisions. Within these phyla, several "typical" freshwater groups have been identified (51, 52, 112, 185, 186). Some of these groups can be numerically dominant and form blooms

when conditions are in their favor (see for example references 57 and 128). For example, filamentous bacteria can dominate bacterioplankton communities when the grazing pressure from bactiovorous ciliates and flagellates is high (86, 127, 153). Phylogenetically, most filamentous freshwater bacteria are affiliated with various cosmopolitan groups of planktonic freshwater *Bacteroidetes*, like LD2, GKS2-217 or CL500-653 (128, 148). Members of the *Bacteroidetes* and *Proteobacteria* exhibit high temporal and spatial variations (18, 119). Both phyla are represented by diverse phylogenetic groups with a wide range of ecological and physiological properties. It has been suggested that the ability of *Bacteroidetes* and *Proteobacteria* to rapidly exploit bio-available organic matter and colonize aggregates could be the main reason for their dynamic distribution patterns (2, 171, 177). In contrast *Actinobacteria* populations do not experience dramatic and frequent variations in abundance over time (119). *Actinobacteria* are omnipresent in humic lakes (18, 60, 173, 174), and freshwater representatives form distinct monophyletic clusters together with sequences recovered from soil environments (174). Most globally distributed freshwater bacterial groups are not closely related to any cultured isolates, and the culture independent studies mentioned above have provided some first insights into the ecological and physiological properties of typical freshwater bacterial populations (see also references 56, 101 and 186). Although these studies have revealed the coarse habitat range for cosmopolitan bacterial groups with respect to several physico-chemical and biotic parameters, still, we cannot make anything more than qualified guesses as to the true mechanisms and factors regulating their distribution in the environment.

Flavobacteria as a class-level model for heterotrophic bacteria

Flavobacteria is an abundant subgroup of the phylum *Bacteroidetes* in many aquatic environments. Although many *Flavobacteria* have been isolated from freshwaters, these isolates are poor representatives of populations detected in culture independent molecular surveys (185). Studies based on culture independent methods have indicated that members of *Flavobacteria* can play a significant role in the degradation of proteins, polysaccharides, and diatom debris in natural environments (28, 80, 129).

Even if *Flavobacteria* are often abundant in freshwater clone libraries (52, 185) quantitative patterns of *Flavobacteria* distributions have only been assessed in a few freshwater systems (51, 127, 152, 153). Most of these studies have focused on effects of grazing on the distribution of the combined *Cythophaga-Flavobacteria* group (127, 128, 152, 153). The effect of resources on the distribution of *Flavobacteria* in aquatic habitats is so far poorly understood.
Previous studies have highlighted the importance of *Bacteroidetes* during phytoplankton blooms in marine and fresh waters (2, 129, 139, 171, 187). One putative explanation for this observation is that some members of the *Flavobacteria* are known to play an important role in the degradation of biopolymers and other types of high molecular weight organic matter (28). Cultured representatives of *Flavobacteria* are physiologically described as organo-heterotrophs that are able to degrade various biopolymers such as cellulose, chitin and pectin (11). Other studies have shown that *Flavobacteria* show a high capacity for growth when dissolved proteins are abundant (28, 129). Also numerous algicidal *Flavobacteria* strains have been isolated from aquatic environments (48, 136, 158, 184).

Vibrio as a genus-level model for heterotrophic bacteria

The genus *Vibrio* is widespread in aquatic environments and several *Vibrio* spp. are potent polymer degraders. Since different *Vibrio* spp. are pathogenic the ecophysiology, diversity and distribution of the genus *Vibrio* in aquatic environments is a public concern. The most well-known and intensively studied species is *V. cholerae*, which causes cholera epidemics worldwide. *V. cholerae* is also involved in other diseases, like ear and wound infection (42, 108, 151). In addition, many other *Vibrio* spp. are also recognized as human pathogens. For example, *V. parahaemolyticus* (113), *V. vulnificus* (68), *V. mimicus* (19), *V. alginolyticus* and *V. hollisae* (121) have all been implicated in water- and seafood-related outbreaks of gastrointestinal infections in humans. There are also increasing reports of lethal wound infections linked to several different *Vibrio* spp. (42). *Vibrio*-related disease is not restricted to humans; e.g., *V. anguillarum* and *V. splendidus* can be potent fish pathogens that are also capable of infecting other marine animals (5), and *V. corallilyticus* and *V. shiloi* can infect and kill corals (9, 95).

The presence of *V. cholerae* and other *Vibrio* spp. together with related diseases in tropical and temperate environments is well documented (6, 27, 61, 62, 65, 164, 165). However, reports about the occurrence, distribution and control of *Vibrio* spp. in northern temperate and boreal waters are scarce (36, 69) even if there are several recent cases of fatal *V. cholerae* non-O1, non-O139 infections in coastal areas surrounding the Baltic Sea (108; see link http://www.smittskyddsinstitutet.se/statistik/vibrioinfektion-exkl-kolera/). The exact sources of the reported clinical cases remained unclear, but they were all linked to either contact with seawater, freshwater or consumption of fish.

It is well known that *Vibrio* spp. are capable of surviving in natural aquatic environments. In general, *Vibrio* spp. tolerate a wide range of salinities (42) and tend to be more common in warm waters, notably when temperatures exceed 17°C (83, 87, 88, 114, 165). Several recent studies have identified various eukaryotic organisms as niches for *Vibrio* spp. in the environment (1, 69, 70, 73, 77, 87, 126, 135, 181). Moreover, in a study of *Vibrio* dynamics in a northern New England estuary, it was suggested that the variance in the concentration of *V. vulnificus* was most significantly explained by dissolved organic carbon (DOC) concentration (84). Another study reported high numbers of *Vibrio* cells during cyanobacterial bloom episodes (122). Exudates released by phytoplankton, as well as feces from zooplankton accompanying algal blooms may constitute potential substrates promoting the growth of *Vibrio* spp. in natural aquatic environments. Yet, the detailed mechanistic understanding of processes underlying these correlations is still incomplete. It is also not clear if these correlations are applicable to a wide range of coastal ecosystems or if they are geographically constrained.

Martinez et al. (110) and Riemann et al. (139) have suggested that the production of particulate matter during phytoplankton blooms create new niches selecting for bacteria specialized in exploiting the energy and nutrients stored in these particles. *Vibrio* spp. should be competitive under such conditions. It has for instance been demonstrated that many *Vibrio* isolates can form biofilms on particles (175) and produce potent extracellular enzymes that degrade DNA, RNA, proteins, lipids, chitin, mucin and many other organic substances (42). Additionally, *Vibrio* spp. are facultative anaerobes, having both a respiratory and fermentative metabolism (42). Since many representatives within this genus are capable of very high maximal growth rates, they may profit from pulsed inputs of organic matter. These widespread physiological features within the genus could provide important clues to the ecological processes responsible for the previ-

ously observed correlations between *Vibrio* spp. and phytoplankton blooms.

The abundance of *Vibrio* spp. in natural environments have mostly been assessed with culture-dependent techniques. It is well known that many *Vibrio* spp. enter viable but non-culturable growth states when exposed to low temperatures or otherwise adverse growth conditions (121, 134, 143). Cells in this state evade detection by culture-dependent techniques since they are incapable of growing on conventional media even if they maintain both viability and pathogenicity (121, 134, 143). Hence, surveys using culture-independent molecular methods are needed to increase our knowledge about the ecology of *Vibrio* spp. in aquatic environments.

Several oligonucleotid based assays targeting the 16S rRNA have been developed to quantify individual *Vibrio* populations in mixed natural communities (36, 62, 63, 131), but the specificity of these assays is limited. To address this gap in methodology, we have developed a *Vibrio*-specific molecular quantification technique that combines quantitative-competitive polymerase chain reaction (qc-PCR) with denaturant gradient gel electrophoresis (DGGE) for the quantification of individual populations within the genus (35).

AIMS of the thesis

In general, this thesis is based on a number of studies where I have followed a "Gleasonian" approach (50) to study single bacterial populations at different taxonomic resolution. In these studies, I tried to identify the niches of biogeochemically important bacterial populations at the class and subclass level, as well as the niche of potential pathogenic *Vibrio* populations in productive aquatic environments.

More specifically, I studied the effects of elevated phytoplankton biomass (i.e. blooms) on bacterial community composition and diversity. This also entailed assessing the spatial and temporal dynamics and the ecological role of individual bacterial populations in eutrophic lakes. Additional focus was on links between community composition and biogeochemically significant C-tranformation processes. The broad aim of the first part of this thesis was to increase the conceptual understanding of the causes and consequences of phytoplankton blooms in productive lakes, and particularly their interactions with heterotrophic bacterial communities and role in the aquatic carbon cycle.

This thesis was not restricted to freshwater environments, since in the second part, the environmental control and ecology of individual *Vibrio* populations was assessed in coastal environments. In addition to an environmental survey of these heterotrophic bacteria, an experiment was performed to assess the effects of temperature and cyanobacterial derived DOM on the competitive potential of various *Vibrio* species.

METHODS

Overview

Papers I, II, III and **V** feature environmental surveys assessing the bacterial diversity and dynamics in productive aquatic environments at different taxonomic levels; e.g. domain *Bacteria* (**paper I** and **paper III**), class *Flavobacteria* (**paper II**) and genus *Vibrio* (**paper V**). Environmental state variables were analyzed in parallel to describe observed dynamics and shifts in these phylogenetically restricted communities. In addition, two studies including a combination of environmental surveys of bacterial community composition and experiments to assess rates relevant for carbon cycling were performed in order to study the biogeochemical role of heterotrophic bacteria in productive lakes (**paper III** and **IV**). Finally, a factorial laboratory experiment addressed the direct effects of temperature and cyanobacterial-derived DOM on growth and accumulation of *Vibrio* populations in coastal water (**paper VI**).

Analysis of biotic and abiotic variables

Water temperature and salinity were measured on site. Total organic carbon was measured by high-temperature catalytic combustion and IR detection in a Shimadzu TOC-5000 total organic carbon analyzer following removal of inorganic carbon by acidification and purging with CO_2-free air (34). Total nitrogen was measured using an Antek 9000 high-temperature-combustion total nitrogen analyzer (Antek Instruments Inc.). Total phosphorus was quantified by an initial hydrolysis of the sample with potassium peroxysulfate (111), and subsequent detection was done using molybdenum blue spectrophotometry (116). Bacterial abundance was analyzed by fluorescence microscopy (133) and flow cytometry (31).
Bacterial production was determined by ^{3}H-leucine incorporation (157) using protein to biomass conversion factors according to Simon and Azam (154). Chlorophyll-*a* was measured spectrophotometrically or by fluoremetry (81).

DFAA were analyzed by high-performance-liquid-chromatography and detected as fluorescence primary amines after derivatization with *o*-phtaldialdehyde as described elsewhere (85, 99). A ^{14}C substrate approach was used to study microbial amino acid uptake kinetics (Bertilsson and Tranvik 1998). The activities of arginine and leucine aminopeptidases were measured by using either L-arginine or L-leucine linked to 7-amido-4-methylcoumarine hydrochloride as a substrate (98).

Molecular tools

The functional roles of many heterotrophic bacterial populations that together form complex communities in natural and engineered ecosystems are often studied only at the community level (e.g. the combined action of all bacteria is usually considered). This is largely because of methodological constraints: the majority of bacteria in natural habitats cannot be studied at the population level by the traditional microbiological methods such as enrichment and subsequent isolation (78). Even when target organisms can be cultivated, properties determined in the laboratory may not necessarily reflect their activities and physiology in the environment, where factors such as resource competition, environmental heterogeneity and fluctuations, as well as predation and other interactions prevail (7, 61).
Methodological advances in molecular techniques (4, 61, 123) have provided new insights into the 'black box' of microorganisms in complex natural communities. Many of the methods are based on DNA cloning techniques and polymerase chain reaction (PCR) using small subunit (SSU) rRNA molecules as population markers. The following suite of culture-independent molecular tools used in this thesis were either adopted to or developed in our laboratory.

Clone library analysis

In **paper I** clone libraries were constructed from four productive lakes sampled during cyanobacterial blooms. Fragments of 16S rRNA genes were PCR-amplified by bacteria-specific primers and cloned into plasmid vectors. High-resolution-terminal-restriction-fragment-length-polymorphism (HR-T-RFLP) was used to distinguish between amplicons from *Cyanobacteria* and other bacterial groups (Fig. 3). Clones were sequenced and phylogenetic trees were constructed in ARB (160) using Maximum Likelihood (43). Bacterial richness and eveness were estimated according to the non-parametric model of

Chao (22) and the reciprocal Simpson´s index (66), respectively. Similar clone libraries of 16S rRNA genes amplified with *Flavobacteria*-specific primers were constructed in **paper II**. Downstream analysis was performed in similar fashion as described for **paper I**.

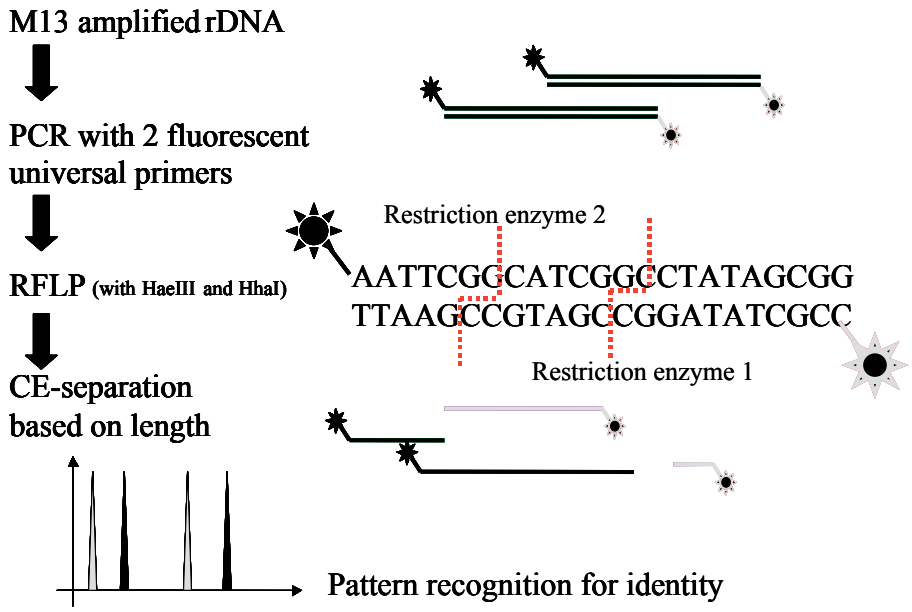

Figure 3. Principles of high-resolution-terminal-restriction-fragment-length-polymorphism (HR-T-RFLP). A first polymerase chain reaction (PCR) is performed using vector primers M13 F and M13 R to amplify the cloned fragments. A 100-fold dilution of the resulting PCR products is used as templates in a second, nested PCR using the bacterial primer 27f labeled with hexachlorofluoroscein and 519r labeled with 6-carboxyfluorescein. Fluorescently labeled PCR products are separately digested with two tetrameric restriction enzymes (HhaI and HaeIII). After inactivation of the enzymes the two digests from the same clone are pooled and the four terminal fragments generated from each clone is sized on an ABI 3700 96-capillary sequencer in GeneScan mode (Applied Biosystems). The resulting T-RFLP patterns are then used to classify clones into operational taxonomic units (OTUs).

Terminal-restriction-fragment-length-polymorphism (T-RFLP)

The bacterial communities were analyzed by T-RFLP community fingerprinting (105) in **paper I** and **paper III**. This method allows the comparison of bacterial communities between various samples. Multivariate statistical analyses, like principal component analysis (PCA), partial least square projection to latent structures (PLS), nonmetric

multidimensional scaling (nMDS) and canonical correspondence analysis (CCA), can be used to reveal relationships of community structure, environmental variables and the function of the community. Also the coupling of single populations with biotic and abiotic parameters can be assessed.
Mind that T-RFLP analysis only detects the most dominant taxa and a simple counting of the number of peaks does not reflect the bacterial richness or diversity of the bacterial community in a sample (46, 103). Several studies have suggested that the structure of most natural bacterial communities are highly skewed, with few abundant and many rare representatives (3, 49, 172). Hence fingerprinting techniques should only be used to compare community composition between samples.

Denaturant gradient gel electrophoresis (DGGE)

In **paper IV**, another community fingerprinting technique, DGGE (117), was applied to separate less abundant bacterial ribotypes from the supposedly dominant cyanobacterial SSU rRNA genes. DGGE bands were excised for subsequent PCR, cloning and sequencing (34), and downstream phylogenetic analysis.

Quantitative PCR combined with T-RFLP

Real-time PCR was performed to determine the relative contribution of *Flavobacteria* 16S rRNA gene copy numbers relative the total bacterial 16S rRNA gene copies. Existing primers were adopted and rigorously tested before application in **paper II**. Parallel to quantitative PCR, the dynamics of individual populations within the *Flavobacteria* community were assessed with T-RFLP fingerprints based on the same *Flavobacteria*-specific primer set (see **paper II** for further description).

Vibrio specific qc-PCR-DGGE

In **paper V** and **paper VI**, a novel qc-PCR method specific for the genus *Vibrio* (165) was adopted and combined with DGGE to discriminate between amplified alleles (e.g. populations; see reference 35). This approach can detect as few as 200 *Vibrio* cells per sample and differentiate between *Vibrio* populations with a single base pair difference in their 16S rRNA gene sequence. The abundance of each *Vibrio* population observed in a sample is determined from the intensity of its corresponding DGGE band using an internal standard and

an experimentally derived titration curve (Fig. 4). The putative identity is determined by migration-based comparison to a reference lane including known isolates.
In **paper 6**, the qc-PCR-DGGE results were also compared to classical cultivation dependent colony-forming unit counts using *Vibrio* specific TCBS agar (32), verifying that culture-dependent techniques greatly underestimate the true abundance of *Vibrio* populations in Baltic Sea water.

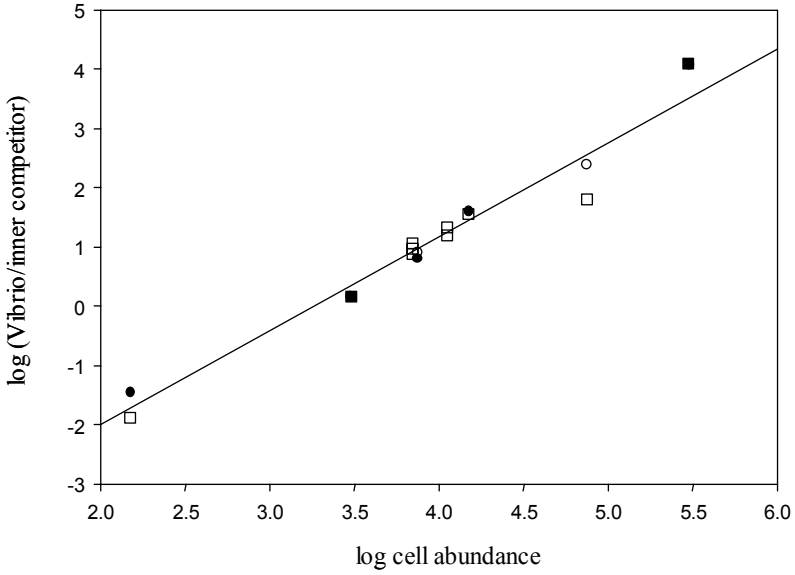

Figure 4. qc-PCR-DGGE standard curve for *Vibrio* isolates in environmental samples (for more detail see reference 35). The ratio of target PCR-product fluorescence (*Vibrio* isolate) to fluorescent signal of the internal competitor (*V. mimicus*, approx. 20 genome equivalents) was plotted against the number of *Vibrio* cells added. *V. cholerae* cells were added to various mixtures of untreated- and filter-sterilized lake water to obtain background concentrations of non-*Vibrio* bacterioplankton ranging from 10^8 to 2×10^9 cells. The different symbols indicate the amount of non-target background bacteria (open square: 10^9cells, closed square: 2×10^9cells, open circle: 5×10^8cells, closed circle: 10^8 cells).

RESULTS and DISCUSSION

Composition of the bacterial community associated with freshwater cyanobacteria

Little is known about the identity of bacteria that inhabit freshwater environments even if these organisms are numerically dominant. The first aim of this thesis was to explore and characterize the composition of bacterial communities in four productive lakes during cyanobacterial blooms. Clone libraries were constructed from various samples and subsequent HR-T-RFLP, sequencing and phylogenetic analyses were performed to reveal the taxonomic relationships of the detected populations.

The estimated OTU richness for *Cyanobacteria*-dominated microbial communities from the four lakes are close to the estimated richness for other lakes and coastal waters (Table 1). These values are also close to the total number of bacterial genome equivalents predicted for lake water based on whole-community DNA:DNA reassociation kinetics (~165 *Escherichia coli* genome equivalents; see reference 140). This suggests that the richness of the bacterial community is not significantly affected either positively or negatively by the conditions created by the cyanobacterial bloom event. The large OTU diversity associated with the cyanobacterial blooms suggests a wide range of unique niches in association with the cyanobacterial blooms.

These niches were apparently occupied by bacteria belonging to various divisions: *Bacteroidetes, Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Planctomycetes*, *Verrucomicrobia, Acidobacteria,* or *Fibrobacteres* (Fig. 5). Five divisions (*Proteobacteria, Actinobacteria, Verrucomicrobia, Planctomycetes and Fibrobacteres*, **paper I**) and one class (*Flavobacteria*, **paper II**) were subject to phylogenetic analyses to study the occurrence of putative clusters of freshwater bacteria that may represent populations characteristic for the environmental conditions created by cyanobacterial blooms.

Many of the 16S rDNA sequences obtained from the four lakes belong to previously described freshwater clusters (52, 185). However, 12 new clusters could be identified within the *Proteobacteria, Verrucomicrobia, Planctomycetes* and *Fibrobacteres* divisions (see for example

phylogentic tree of the α-*Proteobacteria*, Fig. 6). As nine of the 12 newly identified clusters had no closely related sequence in the NCBI database (<95% sequence similarity), these clusters may represent aquatic bacterial populations that are restricted to niches created by cyanobacterial blooms.

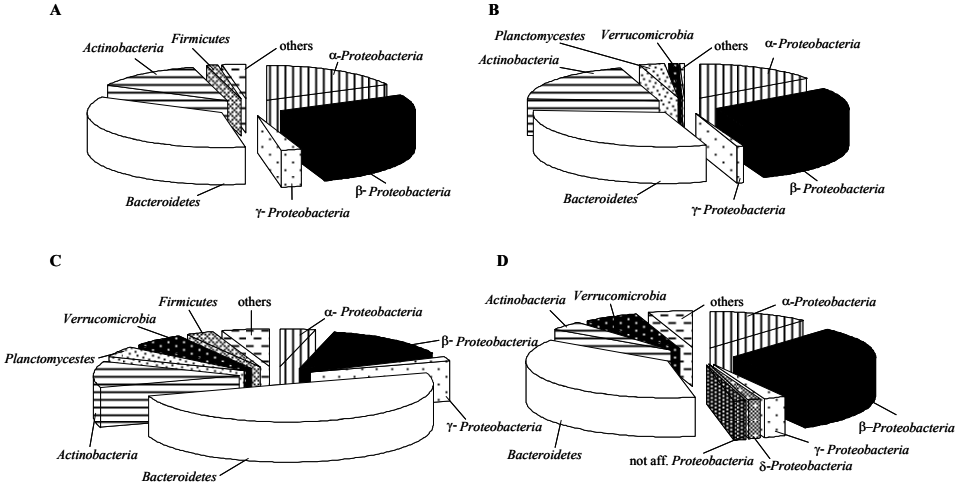

Figure 5. Comparison of the quantitative contribution of clones affiliated with different divisions and subdivisions to the total number of non-cyanobacterial clones in libraries from Lake Ekoln (A), Lake Erken (B), Lake Limmaren (C), and Lake Vallentunasjön (D). Clones affiliated with *Acidobacteria, Fibrobacteres, Chlamydia, Chloroflexi, Thermomicrobia,* and the candidate division OP10 are included in "others".

Coverage analyses indicated that the cyanobacterial bloom libraries contained from 36 to 67% of the total number of ribosomal OTUs that existed in the original water sample from which the libraries were prepared. Hence the universal libraries (**paper I**) do by no means provide a complete inventory of the bacterial 16S rRNA sequences present in the lakes but still represent the most in-depth phylogenetic inventory of freshwater bacteria published.

In **paper II**, the diversity of class *Flavobacteria* was studied more thoroughly using class specific primers when constructing clone libraries. The phylogenetic analysis revealed a wide diversity of *Flavobacteria* that were abundant in the four productive lakes (Fig. 7).

In **paper IV**, the identity and diversity of bacteria attached to *Gloeotrichia echinulata* colonies was studied. Scanning electron microscopy identified a wide variety of bacterial morphologies suggesting a diverse community either attached or closely associated with *G. echinulata* aggregates (Fig. 2a). This was corroborated by sequence

analysis of PCR-amplified 16S rRNA genes in which bacteria affiliated with a number of divisions could be identified: α-, β-, ε-*Proteobacteria, Bacteroidetes, Acidobacteria, Fibrobacteres* and other *Cyanobacteria*, with traces of γ-*Proteobacteria, Fusobacteria, Firmicutes*, and *Verrucomicrobia*.

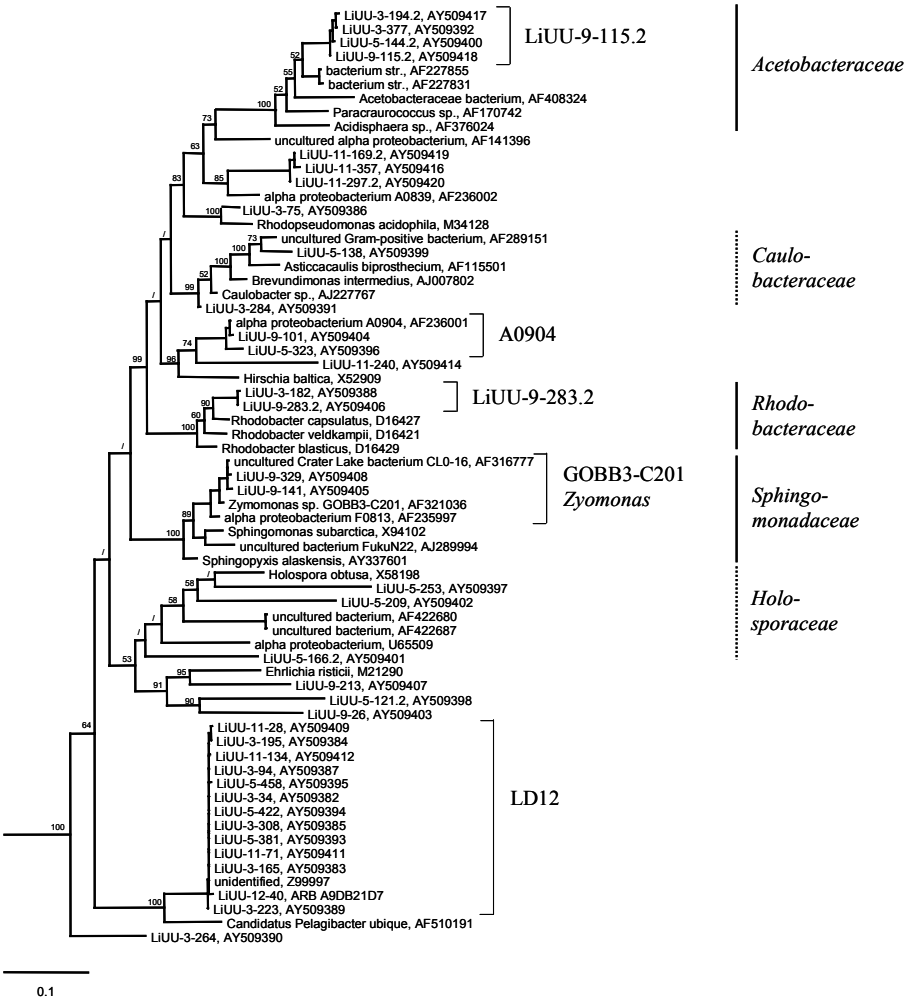

Figure 6. Phylogentic tree inferred by maximum-likelihood analysis of partial 16S rDNA sequences (500-1500 bp) obtained from 16S rRNA clone libraries for Lake Ekoln (LiUU-3-*), Lake Erken (LiUU-5-*), Lake Limmaren (LiUU-9-*), and Lake Vallentunasjön (LiUU-11-*). Bootstrap values at the nodes were calculated using Maximum Parsimony. A slash indicates bootstrap values below 50. Clusters delineated by brackets indicate broader group designations (52, 67, 169, 185) and novel freshwater clusters identified in the present study (LiUU). *Clostridium bifermentans* (AF320283), *Desulfobacterium thermolitrophum* (AJ001049) and *Thermococcus acidaminovorans* (Y15935) were used as outgroups.

To conclude, the results from **paper I**, **paper II** and **paper IV** suggest that numerous niches are created during cyanobacterial blooms either closely or loosely associated with cyanobacterial physcospheres.

A

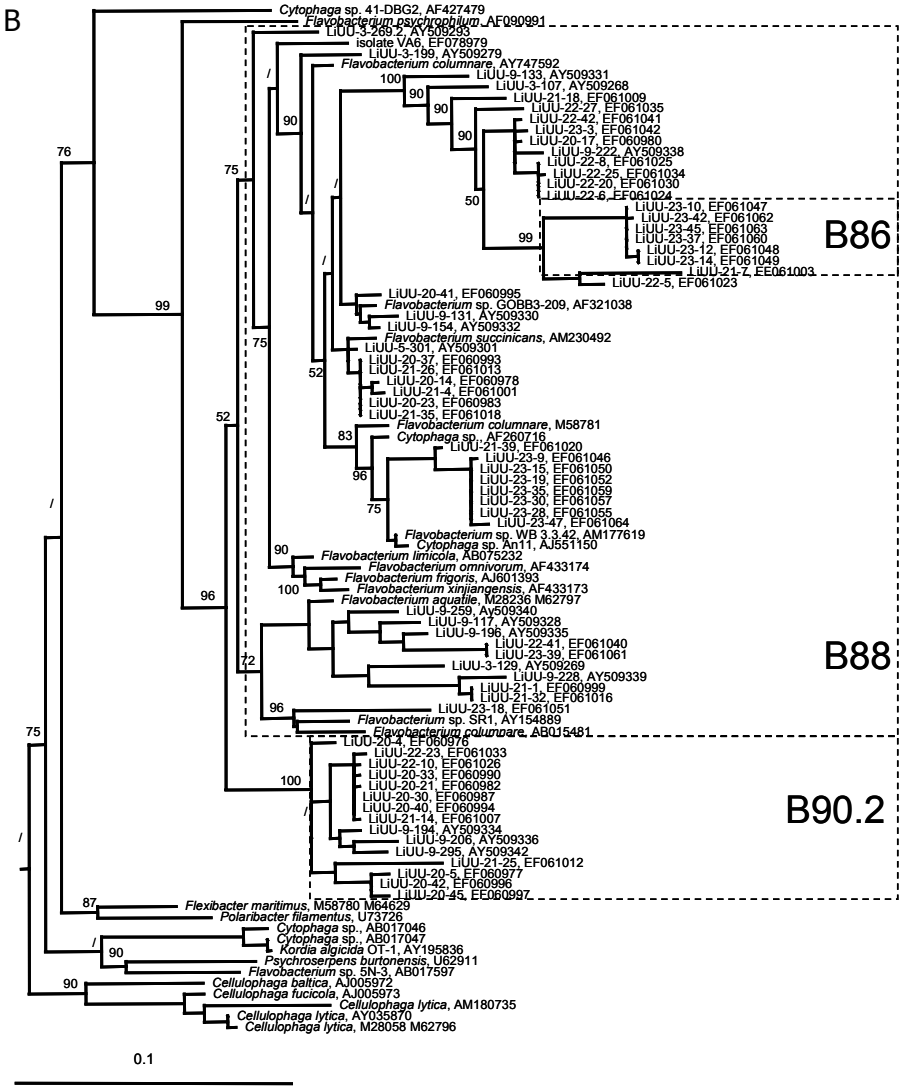

Figure 7A and B. Phylogentic tree inferred by maximum likelihood of partial 16S rDNA sequences (~560 bp) obtained from 16S rRNA clone libraries. Flavobacterial sequences obtained from libraries that were constructed from 16S rDNA fragments amplified with universal primers (27f and 1492r) are indicated by LiUU-3-* (Lake Ekoln), LiUU-5-* (Lake Erken), LiUU-9-* (Lake Limmaren) and LiUU-11-* (Lake Vallentunasjön). 16S rDNA sequences obtained from the clone libraries that were constructed from 16S rDNA fragments amplified with flavobacterial primers (27f and 588r) are indicated by LiUU-20-* (Lake Ekoln), LiUU-21-* (Lake Erken), LiUU-22-* (Lake Limmaren) and LiUU-23-* (Lake Vallentunasjön). *Clostridium bifermentans* (AF320283), *Desulfobacterium thermolitrophum* (AJ001049) and *Thermococcus acidaminovorans* (Y15935) were used as outgroups.

Population dynamics within bacterial communities in productive lakes

In contrast to phytoplankton succession, bacterioplankton succession in productive lakes has rarely ever been studied, even though these mostly organo-heterotrophic microorganisms are major contributors to biogeochemical processes and control water quality (20, 106). **Paper II** and **paper III** aimed to reveal seasonal patterns of bacterioplankton communities in productive freshwater systems. Four productive lakes situated in the temperate zone of central Sweden were studied. Culture-independent techniques (real time PCR, T-RFLP, cloning, and sequence analysis) were applied to study the dynamics of bacterioplankton populations. In **paper II**, the community dynamics were compared to environmental state variables to identify factors regulating populations of freshwater bacteria and elucidate their ecological role in freshwater ecosystems.

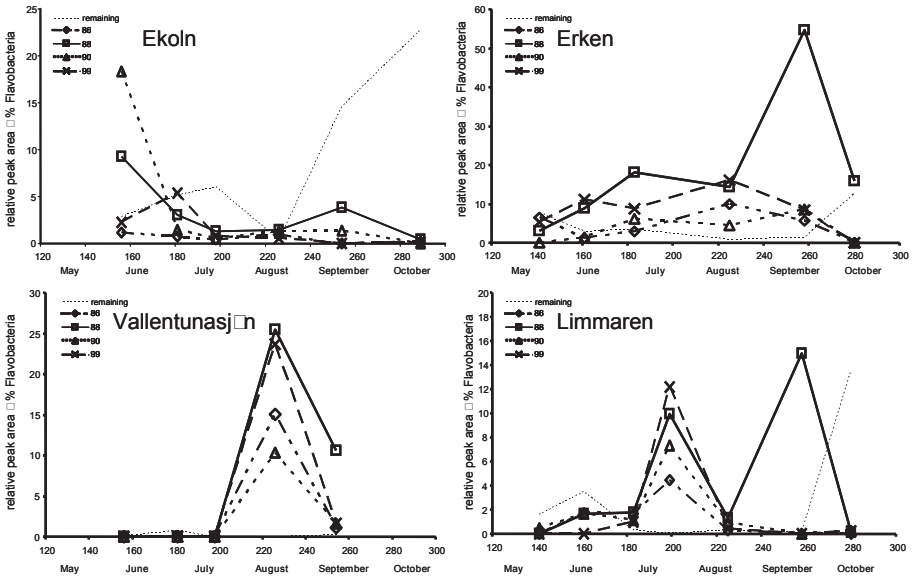

Figure 8. Temporal dynamics of dominant peaks (86, 88, 90, 99 bp) and sum of all other peaks (remaining) from T-RFLP profiles of HhaI-digested PCR-amplified DNA from four lakes. Y-axis represents the relative peak intensity multiplied by the relative abundance of flavobacterial 16S rRNA genes (estimated by quantitative PCR). The affiliation of each T-RF is shown in Figure 7.

The most obvious result that emerged from the seasonal analysis of the bacterial communities was that bacterial populations studied at different taxonomic resolutions, all appear to be highly dynamic in productive lakes (**paper II** and **paper III**). For example, single *Flavobacteria* populations could dominate at different seasonal succession stages and then contribute more than 30 % to the total bacterioplankton abundance (Fig. 8). These blooms of *Flavobacteria* were usually associated with heterotrophic bacterial production maxima (**paper II**, Fig. 9). Absolute numbers of *Flavobacteria* were significantly coupled with bacterial production ($R^2 = 0.48$; $p < 0.001$) and a tight coupling of chl-*a* and bacterial production was also observed ($R^2 = 0.40$; $p < 0.001$). This implies that phytoplankton derived organic matter is one of the main drivers of these summer bacterial communities. It has been speculated that the high abundance of class *Flavobacteria* during phytoplankton blooms and their high proportion within the particle associated bacterial community (177), is due to their ability to degrade complex organic matter, particularly high-molecular-weight dissolved organic matter (DOM) (28, 91).

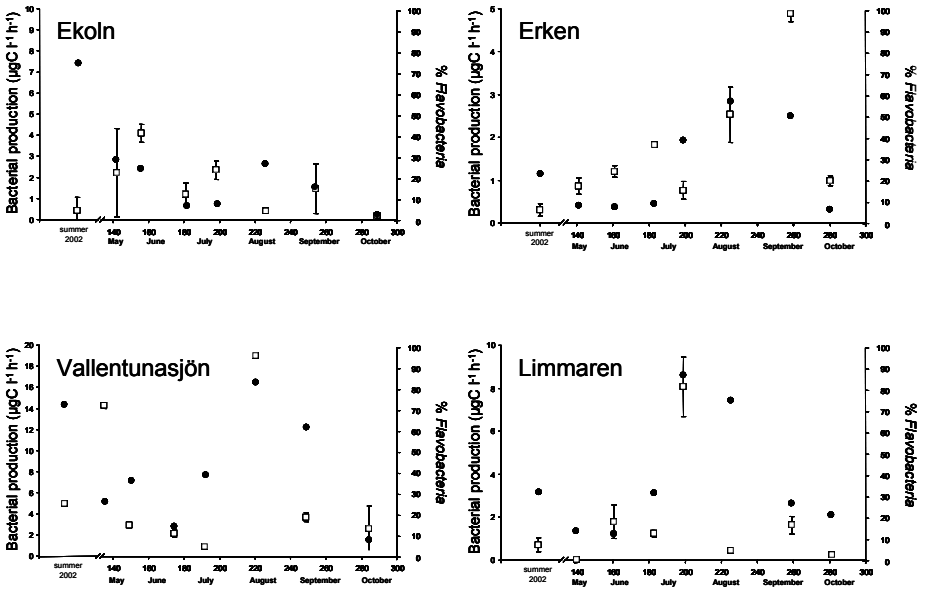

Figure 9. Flavobacterial 16S rDNA as a percentage of total bacterial 16S rDNA (% *Flavobacteria*; open square) and bacterial production (closed circle) in four lakes. All values are averages of triplicate determinations, and the error bars indicate the standard deviations.

However, based on **paper II** and previous studies (129, 130, 139, 171, 187), it is difficult to make any general conclusions about the dynamics of class *Flavobacteria* during phytoplankton blooms since no clear coupling between their abundance and either chl-*a* or phytoplankton biomass could be observed in those studies. About 60 % of the variation in bacterial production and bacterial numbers could not be described by chl-*a* concentrations, suggesting that alternative resources such as allochthonous organic matter could regulate bacterial growth (167). Bacteria are not only bottom-up regulated but also top-down controlled by grazers and viruses in aquatic environments (128, 152, 153, 176). Laboratory experiments and environmental surveys have suggested that certain groups of bacteria are more susceptible to flagellate grazing (82, 152, 153) and viral lysis (150) than others. These studies also suggest that *Bacteroidetes* (Cythophaga-Flavobacteria) dominate the grazing resistant bacterial filaments under enhanced grazing pressure (127, 148). Combining these results, *Flavobacteria* seem to be strong competitors during high predation pressure and high DOM availability.
Cyanobacterial blooms, promoted class *Flavobacteria* (**paper II**) along with the total bacterioplankton community (**paper III**). However, class *Flavobacteria* seemed to be temporarily enriched, particularly during bacterial production maxima. These maxima were dominated by two diverse phylogenetic lineages within the *Flavobacteria* that displayed an inverted dynamic (Fig. 8). Hence it seems that eutrophic lakes harbor highly dynamic bacterioplankton communities with different blooms of *Cyanobacteria* and *Flavobacteria* either alternating or co-occuring.

Biogoeochemical processes mediated by cyanobacteria-associated heterotrophs

Bacteria play a major role in biogeochemical cycles of many elements in aquatic environments, like C, N and P (20, 106). They degrade both organic matter produced by autotrophs within the lake and organic matter imported from the catchment. Especially during phytoplankton blooms, autochthonous organic matter can be the main source of substrates for bacterial growth. Phytoplankton-derived dissolved organic matter is composed of carbohydrates, organic acids, and dissolved free and combined amino acids (45, 183). In **paper III**, the importance of phytoplankton derived organic matter and bacterial processing of this material was studied with special focus on

interactions between DFAA and bacterial community composition. The PLS analyses in **paper III** show that bacterial community composition can explain a large part of the observed variance in community function. Numerous single populations were identified as strong predictors of community function. These might represent microorganisms that are important in utilization of amino acids. These results corroborate the previously described link between bacterial community composition and ecosystem function (for example see references 34, 97, 161).

In **paper IV**, we could show that single populations of a community inhabit different microhabitats, like cyanobacterial colonies and aggregates of organic matter. In general, there appear to be a clear niche partitioning between free-living and particle (or phytoplankton) attached bacteria (55), and these sub-populations exhibit different functions. For example, compared with their planktonic counterparts, bacteria associated with cyanobacterial phycospheres had lower affinity for arginine, used as a model compound to assess uptake of organic compounds.

We also analyzed the role of cyanobacterial–bacterial consortia (*Gloeotrichia echinulata* phycospheres) for net changes in inorganic carbon, primary production and mineralization in Lake Erken (**paper IV**). Colonies and their surrounding phycospheres contributed between 17 (pelagial) and 92% (littoral) to total primary production and phycosphere-associated bacteria contributed between 8.5 (pelagial) and 82% (littoral) to total bacterial secondary production. Over the sampled period, carbon dioxide measurements showed that the phycospheres were net autotrophic in the top layer of the water column, whereas they were net heterotrophic below 2 m depth. Extrapolation of our data to the water column of Lake Erken suggests that microorganisms that were not associated with cyanobacteria dominated CO_2 production at the ecosystem scale during our experiments, as CO_2 fixation was balanced by CO_2 production in the cyanobacterial phycospheres.

Ecology of the genus *Vibrio* in coastal waters: distribution and environmental constraints

In **paper V**, the occurrence of *Vibrio* spp. in Swedish coastal waters was studied. *Vibrio* populations were present in all samples throughout a natural salinity gradient (2 to 30‰) and over the entire sampling season. This suggests that these bacteria are natural compo-

nents of planktonic microbial communities also in the cold waters of Scandinavia. Bacteria of the genus *Vibrio* seem to have a wide environmental tolerance with respect to salinity and temperature. However, water temperatures below 18°C have been shown to limit the abundance of *Vibrio* spp. in coastal waters (83, 87, 88, 114, 165; **paper V**). Nevertheless from our results it is clear that many populations maintain viable populations even under the conditions characteristic for the Baltic Sea, where the annual average temperature rarely exceeds 8°C. In **paper V**, it is shown that bacteria of the genus *Vibrio* were less abundant in the brackish waters of the Baltic Sea (2 to 6‰ salinity). Salinity is known to be an important regulator of *Vibrio* prevalence as different *Vibrio* strains have different requirements for sodium (Farmer and Janda 2005). PLS models (Fig. 10) showed that factors other than salinity, for example phosphorus concentration, and the abundance of microeukaryotes, such as dinoflagellates, also were strong predictors of *Vibrio* abundance in the studied coastal waters. The strong relationship between dinoflagellate abundances and total *Vibrio* abundance implies that dinoflagellates may promote *Vibrio* survival and dispersal in Swedish coastal waters or positively affect the growth of *Vibrio* populations by releasing bioavailable dissolved organic substrates. Several examples are given in **paper V** that suggest that each *Vibrio* population inhabit a distinct niche in the coastal environment and that some *Vibrio* populations may be superior competitors for resources such as nitrogen, phosphorus, or phytoplankton-derived DOM. This is partly corroborated by experimental results in **paper VI**. Here, two of these variables, temperature and phytoplankton-derived DOM, were experimentally tested for their roles as potential growth constraints to four *Vibrio* populations and the natural bacterioplankton community in a factorial-design laboratory experiment (**paper VI,** see also below "The niche of *V. cholerae*"). Temperature has previously been identified as the most important variable regulating *Vibrio* abundances in marine environments when culture dependent methods were applied (75, 83, 87, 88, 107). The experimental results suggest that temperature plays a less important role in determining *Vibrio* spp. abundance than cyanobacterial-derived organic matter in the applied temperature range (12-25°C). This corroborates previous studies that use of DOM derived from phytoplankton (115, 181) can promote the growth of *V. cholerae* and other *Vibrio* spp. (**paper V**) under bloom conditions in natural seawater. One plausible interpretation of the results is that *Vibrio* spp. are capable of out-competing other bacteria by rapidly exploiting the DOM released by phytoplankton blooms.

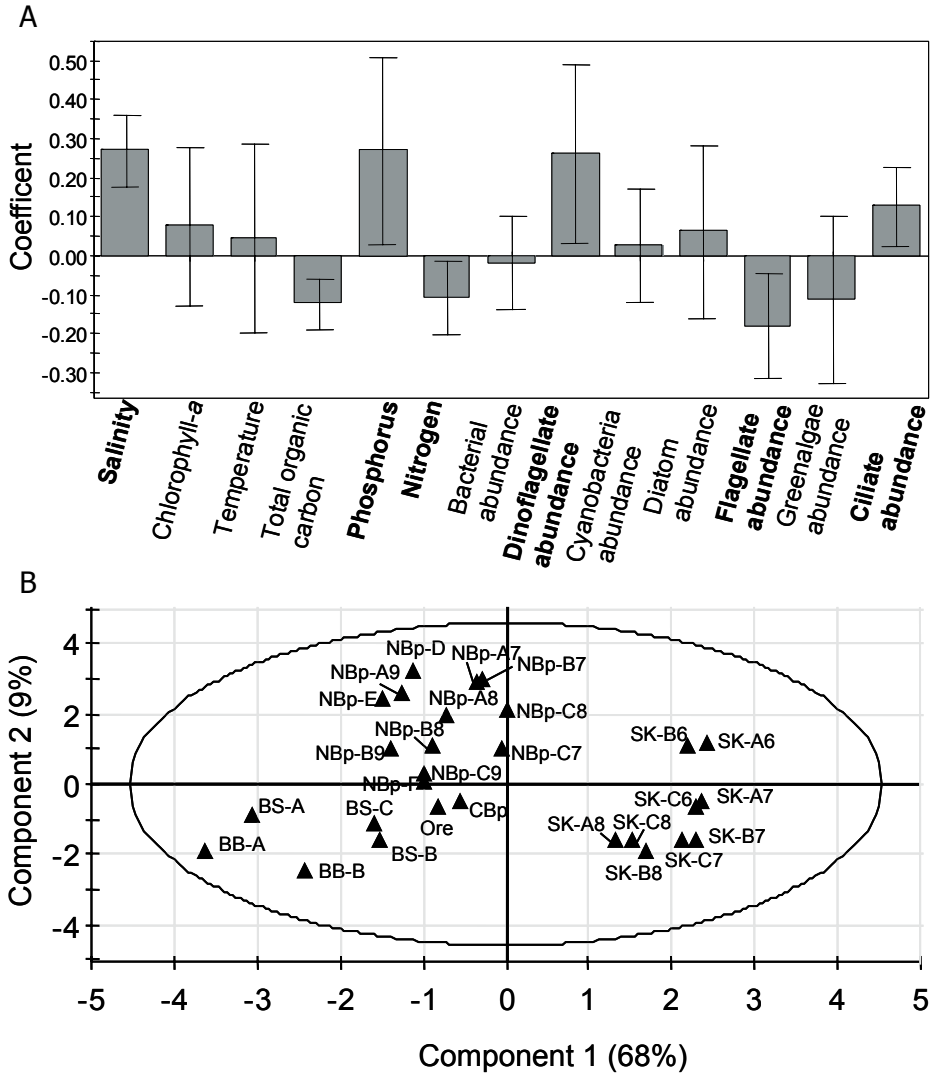

Figure 10. Coefficient plot (A) and score plot (B) of a partial least square projection to latent structures (PLS) model for total *Vibrio* abundance. R^2 = 0.77 (Goodness of fit); Q^2 = 0.57 (Goodness of prediction). Variables significant for the PLS model are indicated in bold (A). SK indicates Skagerrak stations A-C, Ore indicates Öresund station, CBp indicates Central Baltic Proper station, NBp indicates Northern Baltic Proper stations A-F, BS indicates Bothnian Sea stations A-C, and BB indicates Bothnian Bay stations A and B. Month of sampling is indicted by the number.

Another observation of the experiments was that *Vibrio* colony forming unit (CFU) counts were much lower than numbers determined by

the culture-independent method qc-PCR-DGGE, particularly at the end of a 4-day experiment. This might be due to the ability of qc-PCR-DGGE to detect VBNC as well as non-viable *Vibrio* cells. CFU counts actually decreased after 2 days, whereas qc-PCR-DGGE detected dramatic increases in *Vibrio* abundance. This suggests that an increasing number of *Vibrio* cells changed their physiology to the viable but nonculturable (VBNC) state over time in the incubations. Other studies also emphasized a physiological response of the genus *Vibrio* to enter a VBNC state in response to low temperatures and nutrient starvation (for example 17, 21, 115). These studies and **paper VI** suggest that *Vibrio* populations may enter VBNC states under resource limitation or otherwise adverse growth conditions. Hence we should be aware that culture dependent methods are inadequate tools to probe the response of *Vibrio* populations to environmental constraints, since apparent differences in their abundances cannot easily be assigned to either changes in physiology or abundance.
DOM amendment did not only cause an increase in *Vibrio* abundance, but also created a shift in the *Vibrio* community composition in the experiment. The observations in **paper V** and **paper VI** are in agreement with other environmental surveys where massive increases in phytoplankton biomass (i.e. phytoplankton blooms) have resulted in enhanced growth of *Vibrio* spp. (115, 181).

The niche of *V. cholerae*

In **paper V**, the the presence of a *V. cholerae/V. mimicus* population was recorded along the entire Swedish coastline using a culture-independent method (qc-PCR-DGGE; see reference 34). The abundance of *V. cholerae* was also compared to environmental state variables in an attempt to identify the niche and environmental constraints for *V. cholerae* in northern coastal ecosystems.
Since no serotyping was performed, it is not known whether the detected population included strains of recognized pathogenic serogroups such as serogroups O1 and O139. *V. cholerae* strains, including pathogenic serotypes (also other than O1 and O139) (108), have been isolated from estuarine and coastal waters around the globe (104). However, to our knowledge, this is the first time that *V. cholerae* and/or the closely related organism *V. mimicus* have been detected in boreal brackish waters with an annual average temperature of around 5°C and at a latitude as far north as 65°. It is likely that this *Vibrio* population has escaped detection in these waters because previous studies have been biased by the use of culture-dependent

methods. Previous work corroborates that *V. cholerae* can maintain viable populations at temperatures below 15°C and at salinities ranging from 2 to 14‰ in brackish environments (107).
It has been argued that *V. cholerae* proliferate in the environment by forming biofilms (175) and associating with particles as well as organisms (1, 62, 70, 73, 77, 134, 135, 162, 181). However, in our study, 86% (median of 13 samples where a population was detected) of the cells from the *V. cholerae* population remained free living rather than particle or plankton attached (e.g., captured on a 0.8-µm filter). Furthermore, the experimental results in **paper VI** suggest that *V. cholerae* proliferate on cyanobacterial derived DOM when grazing pressure is negligible. This provides strong support for the hypothesis that *V. cholerae* strains can be present as a largely free-living component of coastal bacterioplankton communities and that the common view that *V. cholera*-related disease can be largely avoided by filtration-based removal of larger plankton (74) is not directly applicable to any coastal environment. Our results emphasize that the niche of *V. cholerae* is not as narrow as previously believed and that *V. cholerae* seems to be a strong competitor for phytoplankton-derived DOM.

CONCLUSIONS and PERSPECTIVES

The main results of my thesis can be summarized as follows:

1. The environmental conditions created by the mass-development of *Cyanobacteria* in the productive lakes may produce very dissimilar bacterial communities. In our sampling intensive analysis of 16S rDNA clone libraries, we found no indications that the environmental conditions created by the cyanobacterial blooms lowered the community richness or decreased the evenness of non-cyanobacterial OTUs in the clone libraries (**paper I**). In addition to typical freshwater clusters (52, 112, 185), we were able to identify a large number of novel monophyletic sequence clusters that could represent freshwater bacteria that are characteristic for cyanobacterial blooms (**paper I**, **paper II** and **paper IV**). This descriptive part of my thesis was a first effort to advance our understanding of the identity of heterotrophic bacterial populations in freshwater environments. Isolation and specific rRNA gene–based identification to determine the distribution of these organisms in various freshwater systems holds great promise as strategies to assess the roles/niches of these bacteria *in situ*.

2. A first attempt to study the quantitative distribution patterns of freshwater bacterial populations that are abundant in clone libraries revealed that within class *Flavobacteria,* specific population formed blooms in the four studied lakes (**paper II**). These results and observations presented in **paper III** emphasize that bacterial community composition in productive lakes is highly dynamic and that the regulation of populations is complex. Productive lakes seem to be characterized by marked fluctuations of appearing and disappearing niches, largely as a result of succession of primary producers and fluctuating inputs from the catchment. These high temporal variations may foster an associated heterotrophic community of considerable richness and diversity.
Another interesting question is if single populations inhabit distinct niches in productive aquatic environments. The observed *Flavobacteria* and *Cyanobacteria* blooms imply that productive aquatic environments promote opportunistic microbial populations that can rapidly

exploit readily available organic matter (**paper II**). These opportunistic groups may include potentially pathogenic bacteria as suggested by an environmental survey and a laboratory experiment with bacteria of the genus *Vibrio*. Results in **paper V** and **paper VI** suggest that some *Vibrio* populations are superior competitors during phytoplankton blooms. In contrast to previous belief, we also showed that *V. cholerae* can exist as a free-living compartment of the coastal bacterioplankton in Nordic coastal waters and that this species is thriving on phytoplankton derived dissolved organic matter. Thus, the apparent niche of *V. cholerae* may range from biofilms attached to zooplankton to DOM-rich coastal waters in cold climate.

3. Applying multivariate statistics we were able to predict community functions from the composition of the community. This implies that even if bacterial community functions are shaped by the biotic and abiotic features of the environment, also community composition may regulate ecosystem functions (**paper III**). We could identify a number of populations important for predicting the community functioning. These may represent key-stone populations in the studied productive freshwater environments. Furthermore, the different effects of the population on the model indicate the specific ecological roles of the observed populations.

4. Bacterial species within a certain genus, here exemplified by *Vibrio*, may have dramatically different physiologies and ecological roles (42) and may therefore inhabit different niches in nature. Some species, like *V. cholerae* and *V. mimicus* (**paper V**), exhibit a wider tolerance for key environmental factors and hence exhibit a wider niche breadth than other *Vibrio* spp. with respect to factors such as salinity. This may be caused either by phenotypic plasticity of single genotypes or genetic diversity within a species. For *V. cholerae* both seem to be true: **paper VI** indicates that the niche breadth of a single *V. cholerae* strain can be rather wide; on the other hand previous studies have suggested that different *V. cholerae* strains can cause different types of disease in humans (42, 108). These strains may also inhabit different niches in the environment; i.e. some may form biofilms on zooplankton whereas others may proliferate as free-living bacteria in DOM-rich waters.

One of the major aims of aquatic microbial ecology is to understand the specific role/niches of single microbial populations in the environment. So far, most studies and also this thesis have focused on the occurrence and evolutionary relationships of aquatic microbes.

There is clearly a need to get beyond these studies of microbial community composition by studying the realized niches of single populations, and their role in shaping biogeochemical processes and ecosystem features. Partly, this thesis attempts to give a first insight into the dynamics and niches of an abundant group of aquatic bacteria (*Flavobacteria*) and the genus *Vibrio* that is important from a human health perspective.

Another lesson learned is that we should be cautious about the genetic resolution of the methods used to study microorganisms in the environment. In my opinion, the main problem in current microbial ecology is to study biologically relevant populations with identical evolutionary history and/or genomes. We should be aware that the conclusions drawn need not be true for all members of a population and that single subpopulations may act differently.

Another interesting question that should be addressed is the phenotypic plasticity of single strains and their niche breadth in natural environments. Is the niche breadth of the populations targeted in this thesis and other studies the result of a mixture of genetically distinct ecotypes or phenotypic plasticity within a genetically identical population? It is also important to more thoroughly assess to what extent marker genes actually reveal populations with nearly uniform functions. Recent studies indicate that this is at least not the case for *Prochlorococcus* and certain planktonic *Actinobacteria* (44, 58, 141, 178). We know that different strains of *V. cholerae* can exhibit dramatic differences in pathogenicity as a result of even small genetic variation (42, 108). That points to the importance of intraspecific competition of closely related genotypes in regulating the proliferation of pathogenic genotypes in the environment.

I have three visions for the development of the field of microbial ecology:

(i) Metagenomic libraries and genome sequencing of aquatic bacteria holds great promises to improve our current understanding of processes underlying microbial population dynamics and the role/niche of microorganisms in the environment.

(ii) Future work in microbial ecology should, for example, focus on the metabolic plasticity of single populations, inter- and intraspecific competition and the ecological effects of genome rearrangements.

(iii) In addition to continuously advance the methodologies for studying the abundance and function of bacteria in complex natural communities, microbial ecologists have much to gain from adopting general ecological theories. Microorganisms as a model may be superior models to test certain hypotheses and to advance ecological theories.

ZUSAMMENFASSUNG

Forschung innerhalb der aquatischen mikrobielle Ökologie konzentriert sich sehr oft auf die Ursachen, die die Zusammensetzung der aquatische Bakteriengemeinschaften und ihre Diversität regulieren. Nur wenige Studien haben versucht die Rolle and die Nische einzelner bakterieller Populationen in der Natur zu beschreiben. In meiner Dissertation habe ich neue molekulare Methoden verwendet, um Verbreitung und evolutionären Gemeinsam-keiten von Mikroorganismen in produktiven aquatischen Ökosystemen zu studieren.

Ich fand eine Anzahl neuer phylogenetischer Bakteriengruppen die möglicherweise typisch für eutrophe Seen sind. Eine Gruppe von Bakterien (*Flavobacteria*) scheint eine bedeutende Rolle im Kohlenstoffkreislauf zu haben, da ich während hoher heterotropher Aktivität Blüten von *Flavobacteria* beobachten konnte. Desweiteren variierte der relative Anteil dieser Gruppe an der gesamten Bakterienanzahl weitgehend über den untersuchten Zeitraum. Dies veranschaulicht dass Bakteriengemeinschaften in produktiven Gewässeren sehr dynamisch sind. Mit Hilfe multivariater Modelle konnte ich zeigen, dass mit Wissen über die Zusammensetzung von Bakteriengemeinschaften deren Funktion prädiktiert werden kann. Ausserdem konnte in dieser Arbeit gezeigt werden, dass es grosse Unterschiede in der Zusammensetzung und Funktion zwischen freilebenden und Cyanobakterien assozierten Bakteriengemeinschaften bestehen. Dies legt nahe, dass nicht nur die Umwelt sondern auch die Zusammensetzung der mikrobiellen Gemeinschaft Einfluss auf Prozesse innerhalb des Ökosystems haben kann.

Ich untersuchte auch die Nische potentieller pathogener Bakterien in marinen Küstengewässern. Mit Hilfe einer neuen Methode, die ohne Kultivierungsschritte auskommt, konnte ich zeigen, dass eine *Vibrio cholerae* Population entlang der gesamten schwedischen Küste verbreitet ist. Bisher war unbekannt, dass diese potentiell pathogene Bakterienart auch in borealem Brackwasser mit einem Temperatur-Jahresdurchschnitt von unter 8 Grad Celsius verbreitet ist. Ergebnisse dieser Feldstudie und einem Laborexperiment weisen darauf hin, dass gelöstes organisches Material, welches von Phytoplankton produziert wird, eine bisher unbekante Nische für *Vibrio* Populationen darstellt.

Meine Dissertation und zukünftige Studien über die Rolle von Bakterienpopulationen sollte es ermöglichen biogeochemische Kreisläufe und das Vorkommen pathogener Bakterien im Zuge der globalen Klima- und Umweltveränderungen besser voraussagen zu können.

SAMMANFATTNING

Forskning inom akvatisk mikrobiell ekologi är ofta fokuserad på orsaker och reglering av akvatiska bakteriesamhällens sammansättning och mångfald. Endast några få studier har försökt beskriva enstaka bakteriepopulationers dynamik och deras nischer i komplexa samhällen. I detta avhandlingsarbete har jag använt och utvecklat nya molekylära metoder för att studera olika bakteriers utbredning och deras evolutionära samband i produktiva akvatiska ekosystem. Ett stort antal nya bakteriegrupper som sannolikt är karaktäristiska för eutrofa sjöar identifierades. En grupp av bakterier (*Flavobakterier*) verkar spela en central roll i biogeokemiska kretslopp, och var i de produktiva system som studerades här särskilt abundanta vid förhöjd bakterieaktivitet. Förekomsten av denna bakteriegrupp i förhållande till andra bakterier varierade kraftigt över tid, vilket visar på en betydande dynamik i produktiva vattens bakteriesamhällen.
Genom att använda multivariata modeller kunde naturliga bakteriesamhällens funktion predikteras utifrån artsammansättningen. Det var även stora skillnader i identiet och biogeokemisk funktion mellan frilevande bakterier och de bakteriesamhällen som var associerade till kolonier av cyanobakterier. En generell slutsats av detta är att inte bara miljön utan även det mikrobiella samhällets sammansättning verkar ha ett inflytande på dess samlade funktion i produktiva vatten.
Studierna begränsades inte till sötvatten. Med hjälp av en ny odlingsoberoende metod, var det möjligt att följa olika *Vibrio*-populationers utbredning och dynamik i kustnära vatten. En *Vibrio cholerae* population återfanns längs hela den svenska kusten. Det har tidigare varit okänt att denna potentiellt patogena bakterieart är vanligt förekommande också i boreala kustvatten med en genomsnittlig årstemperatur under 8°C. Denna fältstudie i kombination med laboratorieexperiment i kontrollerad miljö tyder på att löst organiskt material som produceras av växtplankton skapar utrymme för tillväxt av olika *Vibrio*-populationer. Mitt arbete och framtida studier kring bakteriesamhällens roll i naturliga ekosystem kommer att möjliggöra en ökad förståelse av de mekanismer som kontrollerar biogeokemiska kretslopp och vilka faktorer som reglerar förekomsten av patogena mikroorganismer i miljön. Denna information har hög samhällsrelevans mot en

bakgrund av pågående globala klimat- och miljöförändringar och lokal miljöpåverkan.

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