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Microbial diversity in natural environments: focusing on fundamental questions

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Abstract Interactions with Gijs Kuenen and other Dutch scientists have led my lab to fundamental insights into the composition, structure and function of a hot spring cyanobacterial mat community that should influence our thinking about all microbial communities. By focusing on the distribution of molecular sequence variants of predominant mat phototrophs, we have discovered that small-scale sequence variation can be ecologically meaningful. By applying novel cultivation approaches, we have been able to obtain genetically relevant community members and thus to test the hypothesis that closely related sequence variants arose via adaptive evolutionary radiation. By applying the analytical tools of organic geochemistry we have gained insight into the metabolisms of major phototrophic members of the mat community as well as interactions between phototrophic guilds. These observations challenge traditional paradigms about prokaryotic species and cause us to consider evolutionary ecology theory as we develop genome-based methods for high-resolution analysis of the species-like fundamental units comprising microbial communities, and for investigating how such units coordinate the physiological activities within guilds of the community.

Keywords Microbial mats · Molecular analysis · Species · Photosynthesis

Abbreviations

GNSB	green nonsulfur or nonsulfur-like bacteria
DGGE	denaturing gradient gel electrophoresis

Introduction

It is a pleasure to submit this contribution in honor of Gijs Kuenen, a long-time friend and colleague in microbial ecology, as well as other Dutch scientists, many of whom are also associated with the Delft School of Microbiology, who have had a major influence on my research through the years. As I contemplate the impact Gijs has had on my program, I think back to a backpacking trip we made together in the early 1990s to the Edith Baldy Basin in the high country of Montana. We were en route between Hidden Lake and Edith Lake, when Gijs was inquiring as to what my plans were following our initial molecular analyses of well studied hot spring cyanobacterial mat communities. I don't recall my specific

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answer, but it was something along the lines of continuing our analysis deeper and deeper into the molecular diversity of microorganisms of the community, as if on a quest to discover all the community members through their molecular signatures. I also don't recall Gijs' specific reaction, but it was something along the lines of how silly an idea that was. He encouraged me to focus on the predominant and easily detected members of the community, asking fundamental questions about their diversity and ecology, including their contribution to community function. In the intervening years, my students and I have followed this advice and I believe it has led to much more important insights, as Gijs predicted. In this paper, I would like to highlight our progress, paying special attention to ways in which Gijs and other Dutch scientists were involved.

Methods

The variety of methods used to obtain the results presented in this review have been detailed in the primary peer-reviewed papers cited below.

Results

An example of the communities in question, microbial mats in alkaline siliceous hot springs (Octopus Spring and Mushroom Spring,Yellowstone National Park) is shown in Fig. 1. Before my students and I began studying this system as a model for microbial community ecology in 1977, Tom Brock (my mentor), Richard Castenholz and their students had studied the system for a number of years (Brock 1978; Ward and Castenholz 2000). The mat seemed to

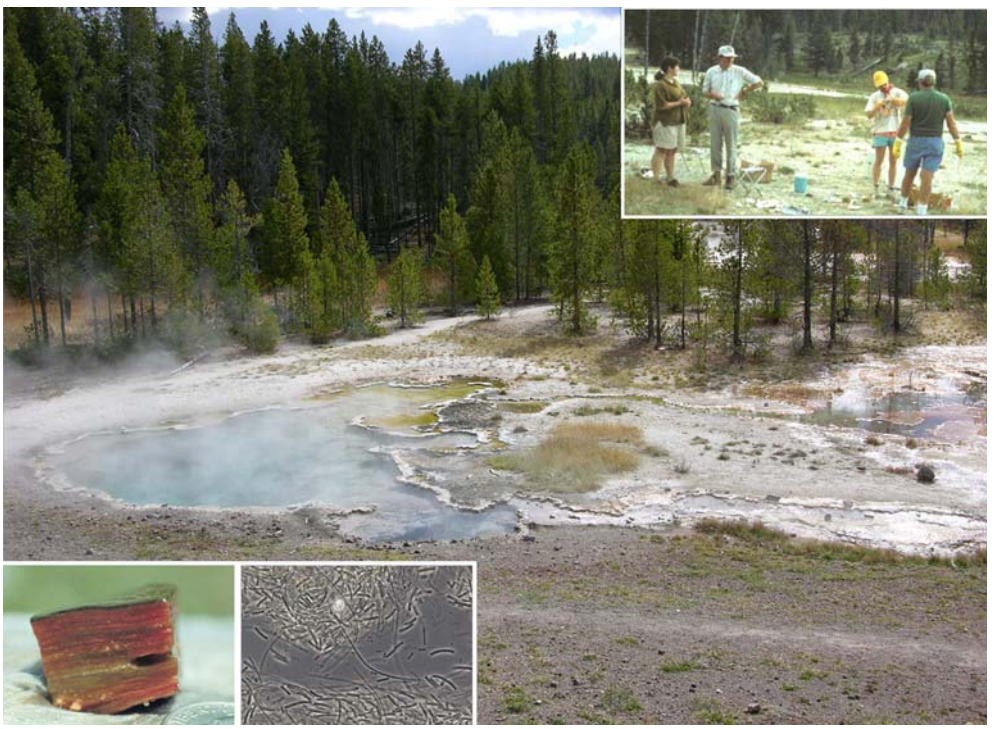

Fig. 1 Landscape view of Octopus Spring, an alkaline siliceous hot spring in Yellowstone National Park. Cyanobacterial mat is visible beneath the inset (upper right) showing Alyson Ruff-Roberts, Gijs Kuenen, Eric Kopczynski and Dave Ward conducting fieldwork in this habitat in the summer of 1991. The top green layer of a mat sample collected at ~ 60°C (inset at lower left) is comprised of sausage-shaped *Synechococcus* and filamentous *Roseiflexus* cells (inset to right of mat image)

be a relatively stable and simple system, constructed by a single morphotype of unicellular cyanobacterium, *Synechococcus*, and a single morphotype of green nonsulfur bacterium (GNSB), *Chloroflexus*. The cultivation from hot spring mats such as these of *S. lividus* (Meeks and Castenholz 1971; Kallas and Castenholz 1982) and *C. aurantiacus* strains (Bauld and Brock, 1973; Pierson and Castenholz 1974) showing little genetic difference reinforced the notion of a simple community composition (Ferris et al. 1996b). By the early 1990s, we had developed 16S rRNA-based cloning and sequencing methods for molecular analysis of the community and had already discovered that the system was more complex than originally thought (Ward et al. 1990, 1992). Several closely related *Synechococcus* 16S rRNA sequence variants were observed, all distantly related to the *S. lividus* 16S rRNA sequence, with variants falling into two clades (A and B clades in Fig. 2A). Similarly, several closely related GNSB-like 16S rRNA sequence variants were observed, all distantly related to the 16S rRNA sequence of *C. aurantiacus* (C-like sequences in Fig. 2B). The GNSB sequences were later recognized as closer relatives of *Roseiflexus castenholzii*, a bacteriochlorophyll *a*-rich filamentous anoxygenic phototroph (Nübel et al. 2002). This paper will focus on the in-depth study of these phototrophic community members, which appeared to be predominant based on the frequency of recovery of sequence variants of these types and on enrichments on highly diluted samples (see below).

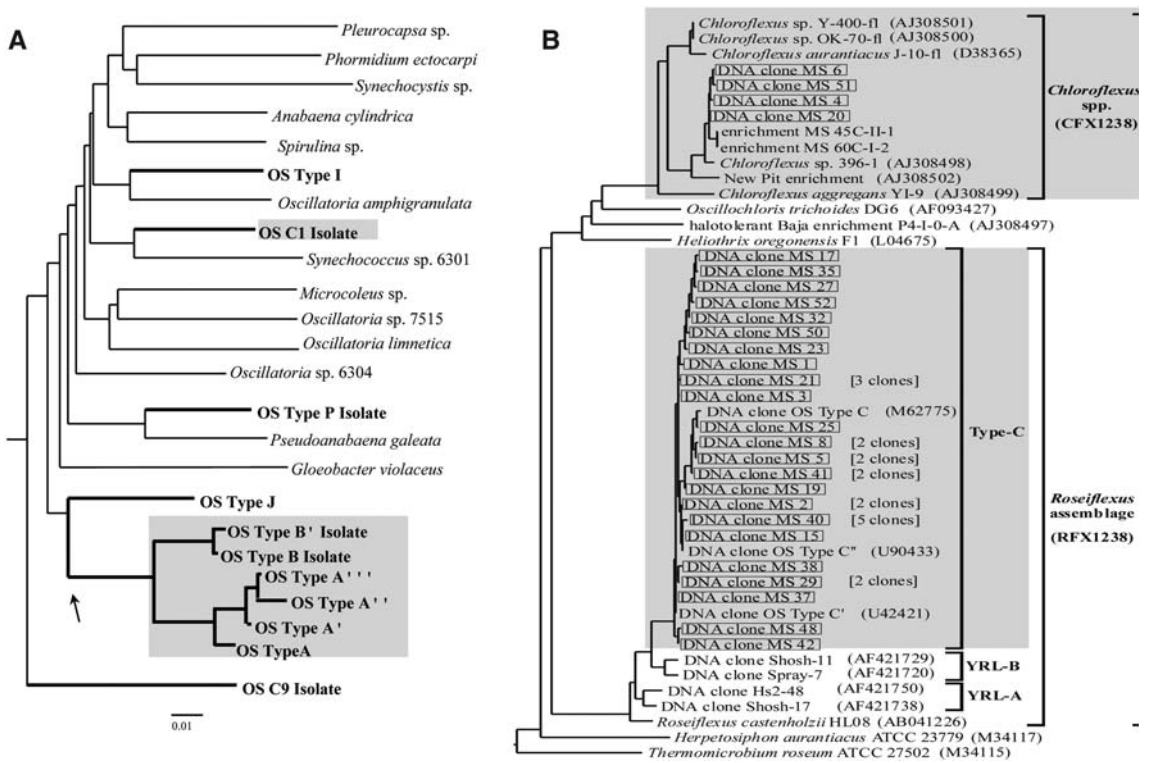

Fig. 2 16S rRNA-based phylogenetic trees of (**A**) cyanobacterial and (**B**) green nonsulfur-like bacterial diversity in the Octopus Spring (OS, thick lines in **A**) or Mushroom Spring (MS, boxed entries in **B**) cyanobacterial mats relative to pure-cultured organisms. Shading contrasts organisms predominant in situ (lower shaded regions) and those readily cultivated (higher shaded regions). Isolate C1 in (**A**) has a 16S rRNA sequence identical to that of *S. lividus*. [Ward et al. 1998 and Nübel et al. 2002]

Community composition and structure

Patterns lead to hypotheses about their causes

We hypothesized that distribution along environmental gradients might give clues to the significance of closely related 16S rRNA sequence variants in the community. Our first approach to distribution analysis was oligonucleotide probing of the 16S rRNAs of both cultivated and uncultivated cyanobacteria (*S. lividus* and genotypes A, B) and filamentous anoxygenic phototrophs (*C. aurantiacus* and genotype C) that were known to us at the time (Ruff-Roberts et al. 1994). Gijs was visiting my lab in the summer of 1991, helping my student, Alyson Ruff-Roberts design and use such probes. This work gave us the first insights that these closely related genotypes (e.g, type-A and type-B *Synechococcus*) were contributed by ecologically distinct community members, since distinct genotypes were found at different temperatures (Fig. 3A) and sometimes at different pHs.

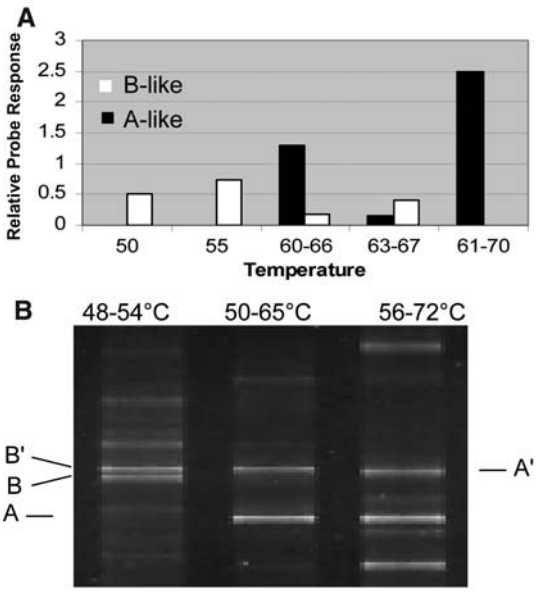

Fig. 3 Patterns suggesting adaptation of Octopus Spring cyanobacteria to different temperatures. (**A**) Response of oligonucleotide hybridization probes targeting 16S rRNA sequences of A-like and B-like *Synechococcus* with mat RNA extracted from different temperature sites [redrawn from data of Ruff-Roberts et al. 1994]. (**B**) DGGE gel of 16S rRNA gene segments PCR-amplified from DNA extracted from mat from different temperature sites; labeled bands were purified and sequenced [from Ferris et al. 1996a]

Shortly thereafter, my lab had the good fortune of collaborating with another Dutch scientist, Gerard Muyzer, who later joined Gijs' department in Delft. Gerard had introduced to microbial ecology a new and powerful method, denaturing gradient gel electrophoresis (DGGE), which separates similarly sized molecules that differ in sequence (Muyzer et al. 1993). DGGE offers greater resolution of closely related sequence variants and is particularly useful for simultaneously observing multiple 16S rRNA sequences in a single sample. Gerard visited my lab to teach my student Mike Ferris DGGE technology. The resulting papers (Ferris et al. 1996a; Ferris and Ward 1997) revealed the presence of even more closely related variants of *Synechococcus* (A′, A″ and B′) and *Roseiflexus* (C′, C″) 16S rRNA sequences (Figs. 2, 3B and 4A). Importantly, DGGE analysis sharpened our focus on how these sequence variants distribute along thermal (Ferris and Ward 1997) and vertical (Ramsing et al. 2000) gradients. It became clear that very closely related 16S rRNA variants (Fig. 4B) exhibited distinct ecological distributions (Fig. 4A). Such evolutionary and ecological patterning lead us to hypothesize that, like many plant and animal species, adaptive evolutionary radiation might explain the origin of the diversity of closely related, yet ecologically distinct populations we were seeing (Ward et al. 1998)(Fig. 4C).

Cultivating the as yet uncultivated

To test the hypothesis that these closely related 16S rRNA sequence variants were from representatives of populations with different ecological adaptations, it was necessary to first cultivate these novel *Synechococcus* and *Roseiflexus* strains. Microbiologists often use the word ''uncultivatable'' to describe the microbes recognized by molecular analysis, but not yet in culture. I prefer the phrase ''as yet uncultivated'' to reflect the optimism (typical of the Delft School of Microbiology) that we will eventually understand how to cultivate such organisms. The extincting dilution enrichment culture approach (i.e., dilution of inoculum before liquid enrichment to avoid overgrowth by rare, but rapidly growing, community members) gave some success in cultivating

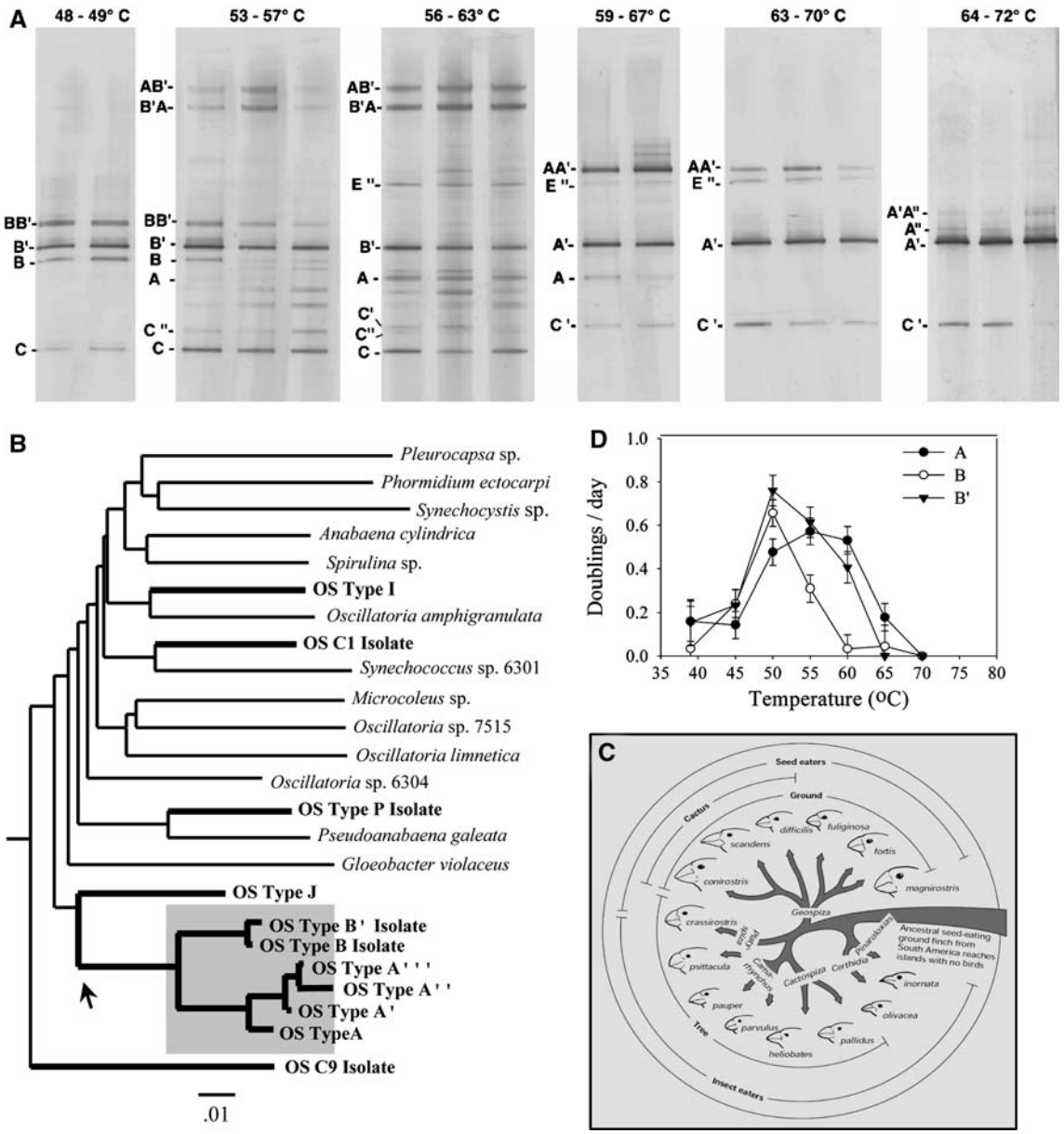

Fig. 4 Patterns suggesting that A/B-type Octopus Spring *Synechococcus* populations evolved via adaptive evolutionary radiation. (**A**) DGGE gel showing progression of B, B′, A, A′ and A″ sequence variants from 48–49°C to 64–72°C temperature sites. [lanes within each grouping are replicate samples from Ferris and Ward 1997] (**B**) Phylogenetic tree shown in Fig. 2A highlighting the descent of these populations from a recent common ancestor. (**C**) Adaptive radiation of Galapagos finches from recent common ancestors into ecologically specialized populations [from Begon et al. 1996]. (**D**) Relationships between temperature and growth rate for *Synechococcus* isolates with B, B′ and A 16S rRNA genotypes that confirm the hypothesis of adaptive differences [from Allewalt et al. 2006]

genetically relevant mat community members, as shown for mat phototrophs by Ferris et al. (1996b) and Ward et al. (1997), and for mat heterotrophs (Fig. 5A) by a visiting undergraduate student from Holland, Sjila Santegoeds, whom Gijs had encouraged to visit my lab (Santegoeds et al. 1996). As shown in Fig. 5A, organisms contributing DGGE bands 1–3 were outnumbered by

organisms contributing DGGE bands 4, 5 and 6 but competitively excluded the more dominant community members unless first removed by dilution to extinction. However, our success in cultivating strains of both major types of phototrophs that are genetically relevant compared to natural populations came as a result of utilizing a unique approach introduced by de Bruyn et al., (1990) in Gijs' department to cultivate iron-oxidizing bacteria. This method involves dilution of a natural sample and filtration onto a nucleopore filter, which is then incubated above a support filter saturated with culture medium (Fig. 5B). The idea is that physical separation of cells prevents overgrowth by less numerically relevant cells with greater fitness for growth on the culture medium. The approach also eliminates the problem that medium solidifying agents may contain toxic materials. In the case of *Synechococcus*, discrete green colonies formed on low-dilution filters (Fig. 5B) and these colonies were successfully subcultured and purified to unicyanobacterial cultures (Allewalt et al. 2006). Representatives of genotypes B, B′ and A were obtained. Although cultures were contaminated by small rod-shaped bacteria, by using direct cell counting it was possible to determine growth rates of *Synechococcus* isolates corresponding to these genotypes (Fig. 4D) and thus to note thermal adaptations consistent with environmental distributions. For instance, the *Synechococcus* B, B′ and A isolates are optimized for growth at 50°C, 50–55°C and 55–60°C and were not observed to grow above 55°C, 60°C and 65°C, respectively, similar to the distributions of these genotypes *in situ* (Fig. 4A). Miller and Castenholz (2000) showed similar results for Oregon *Synechococcus* strains, including an A′-like isolate that showed yet higher temperature adaptation.

In collaboration with Mike Madigan (Southern Illinois University) and myself another Dutch student, Marcel van Der Meer, used the filter cultivation approach to obtain strains of *Roseiflexus* that are genetically relevant to the mat (van der Meer et al., in prep.). It is interesting that these *Roseiflexus* strains did not form discrete colonies, but rather red-colored growth that developed slowly between colonies. This material was subcultured and purified to obtain axenic cultures. The 16S rRNA sequences of these

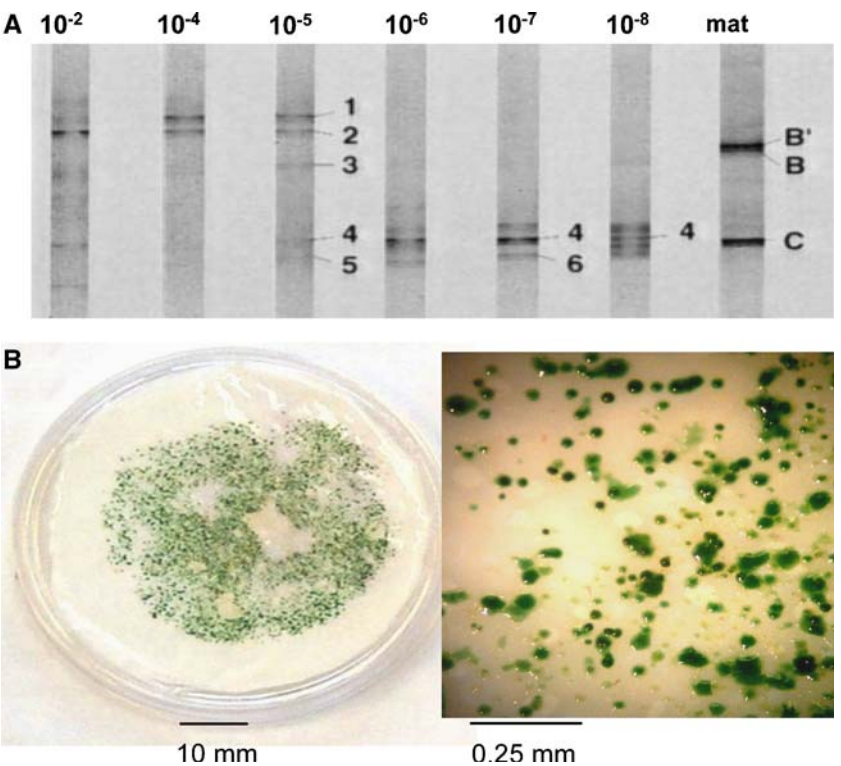

Fig. 5 Cultivation of predominant mat populations. (**A**) DGGE analysis of enrichment cultures for mat heterotrophic microorganisms inoculated with various 10-fold dilutions of mat sample [from Santegoeds et al. 1996]. (**B**) Colonies of *Synechococcus* growing on floating filters

strains were nearly identical to *Roseiflexus* genotypes discovered by Uli Nübel, a student of Gerard's who later became a postdoc in my lab (Nübel et al. 2002). Uli used fluorescent *in situ* oligonucleotide hybridization probing to demonstrate that *Roseiflexus* is a dominant filamentous organism of the mat.

Entering the genomics era

We have recently obtained the genomic sequences of *Synechococcus* strains with genotypes B′ and A as well as metagenomic sequences obtained directly from the mat communities (ongoing research in our NSF Frontiers in Integrative Biology Research program, see http://www.landresources.montana.edu/FIBR/), and a Yellowstone *Roseiflexus* strain (unpublished results of Don Bryant, Pennsylvania State University). These databases are guiding us to yet higher resolution population genetics studies designed to reveal the full complement of ecologically specialized populations in the mat community.

Community function

Together with Dutch organic geochemists, Jan de Leeuw, and Jaap Sinninghe-Damsté (formerly of

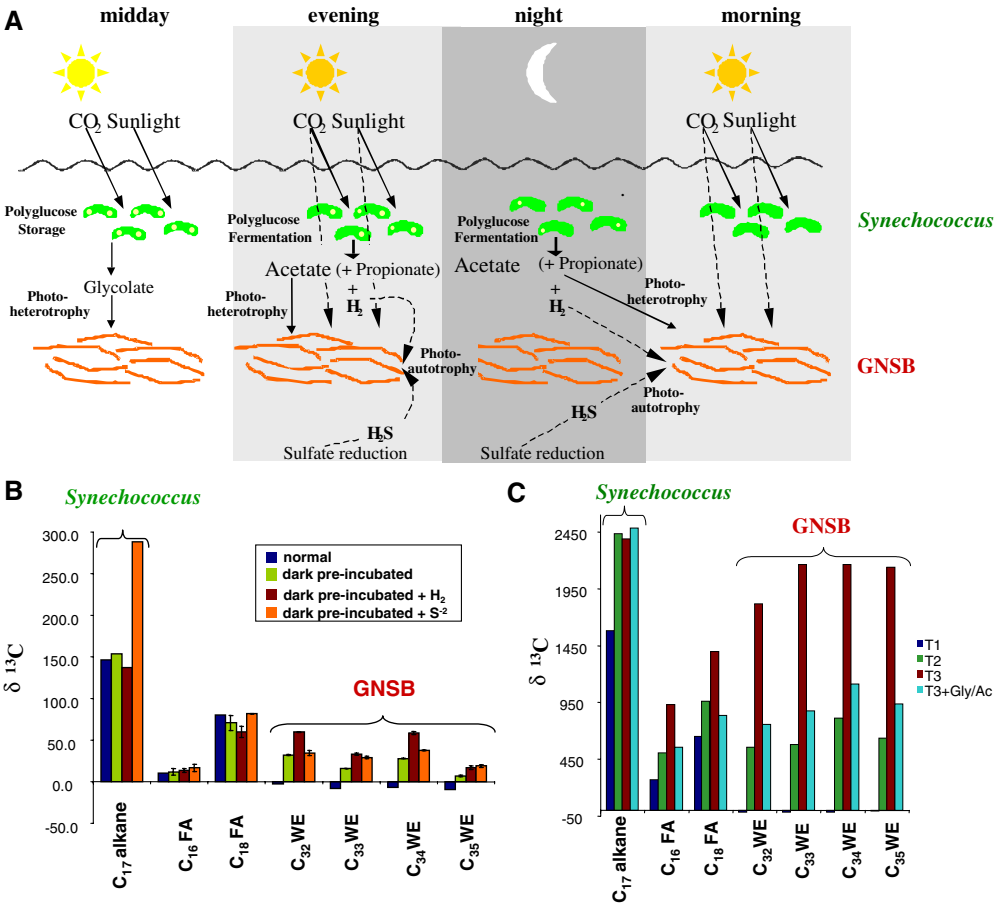

Fig. 6 Physiological processes in alkaline siliceous hot spring microbial mats. (**A**) Model for carbon flow during a diel cycle. (**B**) Incorporation of $^{13}CO_2$ into *Synechococcus* and GNSB biomarkers during a morning incubation period under different incubation conditions. (**C**) Afternoon (T1) pulse-labeling of *Synechococcus* and GNSB biomarkers with $^{13}CO_2$ and subsequent overnight (T2) and morning (T3) redistribution of ^{13}C in the absence or presence (T3 + Gly/Ac) of a mixture of unlabeled glycolate and acetate. GNSB, green nonsulfur-like bacteria WE, wax esters. [from or redrawn from van der Meer et al. 2005]

TU Delft) and Stefan Schouten (all now of the Royal Netherlands Institute for Sea Research, Texel) we have been able to gain fundamental insights into how the major phototrophic community members function in the mat (Fig. 6A). Since mats are considered modern analog of stromatolites, Earth's predominant fossils of the Precambrian Era, our aim is to investigate how the complex reality of mat community members and their interactions may influence geochemical signals that might survive in ancient mats and sediments. Past work had suggested that the filamentous anoxygenic phototrophs (i.e., GNSB-like organisms, such as *Roseiflexus* and *Chloroflexus*) of these mats, which grow especially well photoheterotrophically in culture, were doing so *in situ* as well, consuming low molecular weight organic materials produced and excreted by *Synechococcus*. This hypothesis was supported by observing light-stimulated uptake of fermentation products (Sandbeck and Ward 1981; Anderson et al., 1987) and glycolate (Bateson and Ward 1988) by filaments in the mat.

Photoautotrophy by green nonsulfur bacteria-like community members

Marcel first screened *C. aurantiacus*, *R. castenholzii* and Yellowstone *Roseiflexus* isolates (van der Meer et al., 1999, 2002, in prep.) to discover diagnostic lipid biomarkers that could be used to study the physiology of *Roseiflexus* independently of *Synechococcus*. All of these GNSB-like organisms produce long-chain wax esters, but wax esters of Yellowstone *Roseiflexus* isolates most closely matched those found in the mat (van der Meer et al., in prep.). He then used isotope ratio monitoring gas chromatography-mass spectrometery to study the natural abundance of ^{13}C in biomarkers from these organisms and cyanobacterial mat inhabitants (van der Meer et al. 2000). Based on the argument that ''you are what you eat'', we expected GNSB lipid biomarkers to have $\delta^{13}C$ signatures similar to those of *Synechococcus* lipid biomarkers. As expected, cyanobacterial biomarkers (e.g., nC_{17} alkane) showed $\delta^{13}C$ signatures typical of autotrophs using the Calvin Cycle for CO_2 fixation (e.g., –29.6 to –34.1). However, GNSB biomarkers were isotopically much heavier (–16.4 ‰ to –18.9 ‰) (van der Meer et al. 2000, 2003). One explanation for this apparent anomaly is photoautotrophic metabolism involving CO_2 fixation by the 3-hydroxypropionate pathway, which has been described in *C. aurantiacus* (Herter et al. 2002). This pathway discriminates less between ^{12}C and ^{13}C than does the Calvin Cycle and thus leads to production of organic matter with a heavier ^{13}C signature (Holo and Sirevåg 1986; van der Meer et al. 2001). We were able to find evidence suggesting the potential for autotrophy through incorporation of $^{13}CO_2$ into wax esters during morning hours, when H_2S (and possibly H_2) accumulate as products of fermentation and sulfate reduction in the mat photic zone (van der Meer et al. 2005). This activity required preincubation to allow labeled substrate to diffuse throughout the sample, and was stimulated by addition of H_2 and H_2S (Fig. 6B).

Photoheterotrophy by green nonsulfur bacteria-like community members

We have recently been exploring a second possible explanation for the heavier $\delta^{13}C$ signatures of *Roseiflexus* biomarkers. My NIOZ colleagues were aware that sugars produced by cyanobacteria have heavier $\delta^{13}C$ signatures than do lipids (van Dongen et al. 2001). We had discovered in earlier work that polyglucose is the main photosynthetic product in the mats (~84% of fixed $^{14}CO_2$), and that this polyglucose is fermented primarily to acetate and CO_2 under dark anaerobic conditions (Nold and Ward 1996)[1]. Furthermore, as mentioned above, we knew that acetate is photoincorporated into filamentous cells in the mat. We hypothesized that transfer of isotopically heavy sugar fermentation products, especially acetate, between *Synechococus* and GNSB could explain the heavier $\delta^{13}C$ of GNSB lipid biomarkers. We first demonstrated that the $\delta^{13}C$ of sugars in the mat was heavy enough (–11‰) to explain the

[1] While pursuing a Ph.D. in my lab, Steve Nold mentored Sjila Santegoeds; after receiving his degree he did a postdoctoral in Holland with Riks Laanbroek.

heavy GNSB lipids (–16.4 ‰ to −18.9 ‰) (van der Meer et al. 2003). We then attempted to track the flux of carbon between *Synechococcus* and GNSB, as follows (Fig. 6C). We (i) conducted an afternoon labeling of mat samples with $^{13}CO_2$, which appeared to label mainly *Synechococcus* lipids (Fig. 6C, T1) and, based on the earlier ^{14}C labeling studies, should have resulted primarily in production of ^{13}C-labeled polyglucose, (ii) removed unreacted $^{13}CO_2$ and (iii) allowed the samples to incubate overnight. ^{13}C appeared in GNSB wax esters by morning (Fig. 6C, T2) and the extent of labeling increased through the morning hours (Fig. 6C, T3), suggesting transfer from *Synechococcus*. Interestingly, addition of an unlabeled acetate/glycolate mixture (intended to trap suspected labeled intermediates) prevented the increased labeling during morning hours (Fig. 6C, T3 + gly/ac), suggesting that acetate and/or glycolate may be specifically involved as intermediates in the exchange of ^{13}C between *Synechococcus* and GNSB. Overall, these results are consistent with the hypothesized night-time fermentative metabolism of *Synechococcus* and photoheterotrophic incorporation of fermentation products by GNSB in the morning.

The relative importance of these two mechanisms has not yet been resolved. Nearly equivalent rates of C uptake via autotrophy and heterotrophy (estimated from ^{13}C-acetate incorporation experiments) were observed, however, these estimates are subject to many experimental limitations (van der Meer et al. 2005). Furthermore, the durations of photoautotrophic and photoheterotrophic metabolisms during the diel cycle are unknown. New approaches enabled by genomic data are now being taken to attempt to learn more about the timing of these processes during a diel cycle. Steunou et al., (2006) have demonstrated that in situ expression of *Synechococcus* genes involved in photosynthesis and fermentation follows a pattern that is entirely consistent with a shift from daytime photosynthesis to night-time cyanobacterial fermentation. We plan to conduct parallel studies of *Roseiflexus* gene expression, enabled by the new genome sequence that should reveal when its photoautotrophic and photoheterotrophic metabolisms occur in the mat as well.

Discussion

The advice of Gijs Kuenen to focus on questions of fundamental importance, and on major players/dominant organisms, has led us to new insights into the composition, structure and function of microbial communities. Clearly, the development of techniques for molecular analysis of microbial diversity has revolutionized microbial ecology. Molecular analysis has revealed a vastly different view of microbial community ecology than was provided by traditional methods (e.g., microscopy and cultivation). More importantly, it has enabled us in recent years to begin to take a population-based view of microbial community ecology similar to the view that plant and animal ecologists have developed.

As a community ecologist I view the most pressing concerns as being (i) which microorganisms comprise a community, (ii) how they are structured within the community and (iii) how they control the functioning of the community. Molecular approaches have identified the populations that are truly dominant in microbial communities and have revealed the vast genetic differences between these and the more readily cultivated microorganisms. In our system, in both photosynthetic guilds (oxygenic and anoxygenic), microscopy oversimplified diversity and cultivation methods reinforced this oversimplified view. Furthermore, more often than not, sets of closely related molecular variants are observed in molecular analysis of microbial communities. This raises the obvious question of the importance of small-scale molecular diversity.

To truly understand community composition and structure we need to know whether, and if so how, molecular variation is linked to species variation. I use the word ‘‘species’’ intentionally for two reasons. First, biologists studying communities of plants and animals consider species as the fundamental units of which communities are comprised, the populations that will uniquely rise and fall when the environment changes because they are adapted to distinct niches (Mayr 1982) Second, use of the word species evokes debate about the subject, which I believe is healthy, especially in microbiology today. I have argued against traditional prokaryotic species concepts

and for more natural species concepts (Ward 1998, 2006). This argument was first based on evolutionary and ecological patterns that reveal that closely related 16S rRNA variants of *Synechococcus* represent distinct ecological populations. As I have suggested in a current review (Ward 2006), such patterns have been commonly observed in molecular microbiology studies of various microbial communities. Here, I highlight the work of Uli Nübel, Gerard Muyzer and colleagues (Nübel et al. 2000), which demonstrated that members of evolutionary clusters of cyanobacterial 16S rRNA variants detected in hypersaline microbial mats exhibit unique distributions along a salinity gradient, at least in part due to adaptations to salinity (Fig. 7). As mentioned above, in botany and zoology such patterns have been interpreted as adaptive evolutionary radiations of species (e.g., Galapagos finches, Hawaiian silversword plants, see Ward et al. 2002). A popular view in microbiology is that we can only be certain that populations whose 16S rRNA sequences are at least 2–3% different are distinct species (Stackebrant and Goebel 1994; Goodfellow et al. 1997; Venter et al. 2004). However, this does not seem reasonable, given the obvious adaptive differences of populations exhibiting $<1\%$ 16S rRNA difference, and, in some cases, of populations with identical 16S rRNA sequence, but apparently unique ecologies (Ferris et al. 2003). It is important to realize that such molecular cutoff's result from quantification of the genetic differences among strains of named prokaryotic species, hence, reinforcing our faith that named species are true species (Cohan 2002).

In a current review (Ward 2006) I present the argument that there is much to be gained by considering how macrobiologists think about

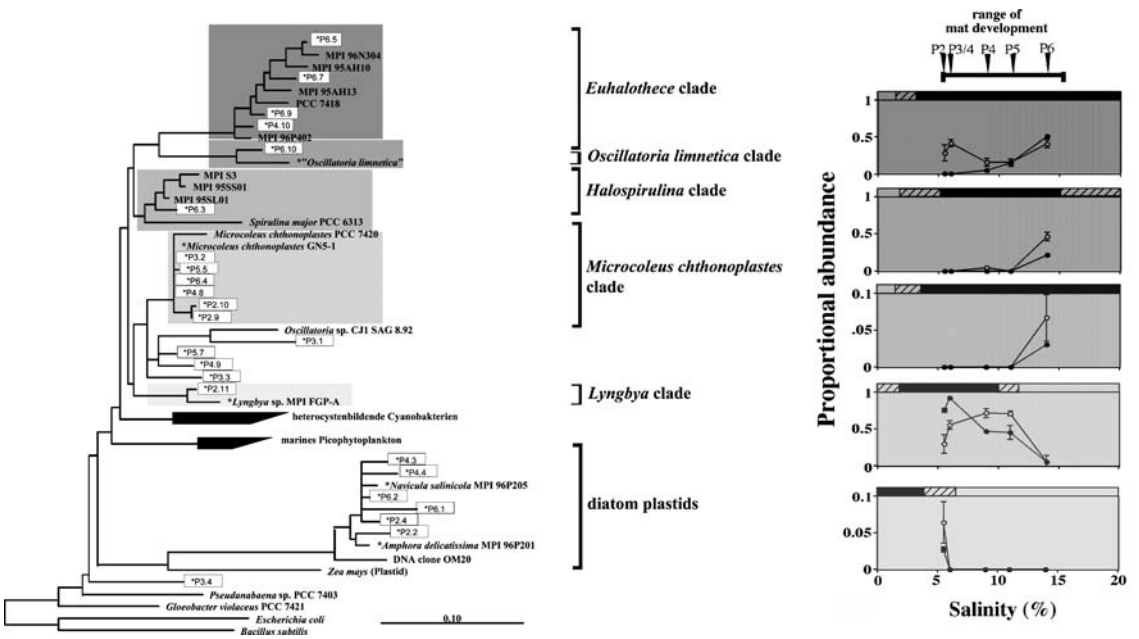

Fig. 7 Patterns suggesting evolutionary radiation of cyanobacteria with different salinity adaptations in Guerrero Negro, Baja California Sur, Mexico salt evaporation pond microbial mats [redrawn from Nübel et al. 2000]. (**A**) Cyanobacterial phylogenetic tree based on 16S rRNA sequence variation, showing relationship of sequences retrieved as DGGE bands from ponds of different salinity (boxed entries with pond designations P1–P6) to sequences of representative cyanobacterial cultures. Shading of decreasing intensity (top to bottom of tree) is used to highlight 5 major phylogenetic clades. (**B**) Proportional abundances (○, microscopic counts; ●, DGGE band intensity) of the members of each clade highlighted in (**A**) as a function of salinity of different ponds. Dark and hashed horizontal bars above each distribution plot exhibit ranges of optimal and suboptimal growth rate ranges, respectively, for isolates from each clade. Abundance diagrams in (**B**) are highlighted using the same intensity scheme used in (**A**)

species. The argument is based in large part on interactions with Fred Cohan (Wesleyan University) with whom I spent a recent sabbatical leave. His course ''Origins of Bacterial Diversity'' (available at http://www.landresources.montana.edu/FIBR/ and which makes a wonderful graduate-level tutorial) and approaches (e.g., Cohan, 2002; Gevers et al. 2005) organized my thinking about evolutionary ecology theory. Periodic selection theory is based on the premise that species are populations whose individual members exhibit variation, but occupy the same niche (Fig. 8A). Occasionally, natural selection (more precisely periodic selection) favors a most-fit variant, squashing diversity within the population; the survivor founds a new population that, with time, redevelops diversity as neutral mutations accumulate within new individuals. A series of periodic selection events provides the cohesive force holding the ecological population together as it evolves within the niche. The evolution of a variant that is capable of occupying a new niche, leads to the evolution of a new ecological population that is free to diverge from the parent population because periodic selection affects only the organisms within a single niche. Given sufficient time, the new population diverges through its own series of periodic selection events to form a new species. Periodic selection theory thus predicts the evolution of terminal evolutionary clades that unite individual variants into species-like ecologically specialized populations, matching the empirical observations we (Ward and Cohan 2005) and others (Ward 2006; Nübel et al. 2000) have made. Interestingly, both empiricists and theorists have used the word ''ecotype'' to describe these species-like populations (Ward and Cohan 2005).

As I see it, the detection of species in a microbial community will require development of methods with sufficient molecular resolution to observe variation among individuals within terminal evolutionary clades. Cohan has pointed out that the neutral mutations that accumulate uniquely in the genes of diverging populations may provide a means of detecting unique ecological populations (Fig. 8B), at least those that have diverged sufficiently to accumulate enough unique neutral mutations. We must then rely on

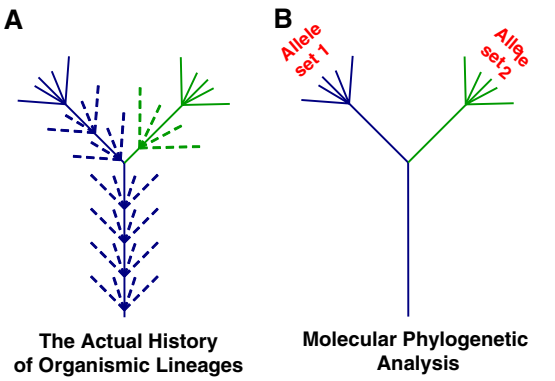

Fig. 8 Periodic selection model for the evolution of prokaryote species populations. (**A**) proceeding upward from the base of the tree, a blue fan of diversity is squashed (dashed lines indicate extinction of individuals) due to the selection of a single most-fit variant (solid line), which diversifies again (2nd fan); a series of periodic selection events provides a cohesive force holding the population together. The divergence of a new population (green) occurs when a variant acquires the ability to occupy a new niche and is thus immune from the periodic selection events affecting the parental (blue) population. Eventually, the two populations diverge into two distinct clusters corresponding to two distinct ecological populations. (**B**) Modern phylogenetic tree showing distinct sequence clusters with unique sets of allelic variants

evolutionary ecology theory to help us predict how individual molecular variants group into clades. Cohan is developing theory-based evolutionary simulations to do this. One method (Cohan 2002), is based on multi-locus sequence typing, a high-resolution molecular approach used by microbial population geneticists to demonstrate the existence of many species-like populations within named bacterial species (Maiden et al. 1998). Cohan and I, together with other collaborators are currently working to develop other approaches, based on genomic analysis (http://www.landresources.montana.edu/FIBR/). These approaches will be used to test various models of speciation (Ward and Cohan 2005; Gevers et al. 2005), including models that consider horizontal gene flow ''rare, but promiscuous'' (e.g., periodic selection model) or so frequent that it would prevent the formation of species through the divergence of ecologically specialized populations, as suggested by Fig. 8. I find it hard to imagine how a high-frequency horizontal gene flow model of species could be

reconciled with the frequent observations of evolutionary and ecological patterning referred to above. If horizontal gene flow erased the genetic signatures of ecologically distinct populations as they form, why would such signals ever be left in the phylogenetic record?

This goal of understanding community structure depends on identification of the fundamental species-like units of which a community is comprised. However, we are already gaining insight through studies of conserved molecular markers. At present, we may be probing sets of species-like units rather than the individual species-like units themselves, but we can at least begin to observe the kinds of adaptations (e.g., to temperature, light, pH and salinity) that help us understand the diversity we see. Given the enormous diversity that has already been revealed by studying slowly evolving molecular markers (e.g., 16S rRNA), I marvel at the multitude of adaptations that might have to be invoked to explain the full ecological diversity of microorganisms in nature. I see as one of the big challenges for the future unraveling the features of the abiotic and biotic environment that have influenced the evolutionary trajectories of species-like populations. This will not be a simple problem to resolve. We must keep in mind Hutchinson's concept of niche as *n*-dimensional hypervolume (Hutchinson 1957). Possible first examples of the complexity of niche defnition appear to be emerging from comparative genomic analysis of closely related cyanobacterial species-like populations. For instance, marine *Prochlorococcus* populations that exhibit distinct adaptations to light and hot spring mat *Synechococcus* populations that exhibit distinct temperature adaptations, have also been found to exhibit differences in ability to acquire and store inorganic nutrients, consistent with possible spatial differences in nutrient availability in marine and geothermal habitats (Rocap et al. 2003; unpublished research in our NSF Frontiers in Integrative Biology Research program).

The main insight gained to date from our studies of phototroph function in the mats may be to ''expect the unexpected''. While we had thought of native *Synechococcus* populations as actively growing and dividing, it appears that they are instead in an active, but slowly growing (or perhaps nongrowing) physiological state (Nold and Ward 1996). In such a high-density population (i.e., ~10^{10} *Synechococcus* cells/ml) it is easy to speculate that this phenotype might be under quorum sensing control. As opposed to balanced macromolecular synthesis, daytime photosynthesis leads mainly to polyglucose accumulation and polyglucose is fermented at night. The diel rhythm of photosynthesis and fermentation in *Synechococcus* may well be under circadian control (ongoing research in our NSF Frontiers in Integrative Biology Research program). The routing of carbon via sugar biosynthesis has an interesting effect of the ^{13}C signatures of organisms in the mat, since sugar biosynthesis exhibits a lower selection of ^{12}C over ^{13}C than does lipid biosynthesis. This heavier ^{13}C signature may be transferred from oxygenic photoautotrophs to photoheterotrophically metabolizing filamentous anoxygenic phototrophs that assimilate isotopically heavy fermentation products. Since GNSB cell components appear to be more persistent than cyanobacterial cell components (van der Meer et al. 2000), this signal may have a potential to survive and become a part of the organic geochemical record. *Roseiflexus* and *Chloroflexus* are best known for their photoheterotrophic metabolism and, in fact, only *Chloroflexus* has been grown photoautotrophically (Holo and Sirevåg 1986; van der Meer et al. 2001; Hanada et al. 2002; van der Meer et al., in prep.). Nevertheless, it appears that photoautotrophic metabolism is possible in morning (and perhaps evening) hours, when anaerobic processes lead to the accumulation of possible electron donors in the dimly lit, anoxic photic zone. Clearly, we have much to learn about the physiology of these major mat phototrophs (e.g., can *Roseiflexus* conduct photoautotrophic metabolism?), but genomics is beginning to provide a more global appreciation for possible metabolisms. A striking example is the discovery that *Synechococcus* A and B′ isolates possess and express (Steunou et al. 2006) *nif* genes in situ in concert with the occurrence of nitrogen fixation. This would not be such a surprise were it not for the fact that previous workers failed to detect nitrogen fixation in these mats (at these temperatures; Stewart, 1970; Wickstrom 1980), perhaps because their incubations did not

take into consideration that the mat environment is anaerobic at night (Revsbech and Ward 1984). Our research team is conducting targeted gene expression studies of specific *Synechococcus* and *Roseiflexus* metabolisms and is developing microarrays with the aim to determine in situ spatiotemporal patterns of expression of all genes in these organisms.

By combining a population-based analysis of molecular microbial diversity with functional analysis, we are beginning to understand the value of diverse ecological populations to microbial communities. In the mat systems we study, Michael Kühl (Marine Biological Laboratory, University of Copenhagen) has observed evidence that multiple, co-existing cyanobacterial populations, differently adapted to temperature, might increase the resilience of oxygenic photosynthesis to variations in temperature (see Fig. 6 in Ward et al. 1998). This suggests that intra-guild diversity may stabilize guild activities and provides a means of organizing our thinking on how community structure is linked to community function.

The very sophisticated tools of organic geochemistry are beginning to help us see that the complex realities of microbial community composition, structure and function do matter to the resulting geochemical traces that might provide relicts that could be used to unravel the chemical fossil record of microorganisms in the Precambrian Era. However, even these techniques only allow us to evaluate guild processes, rather than the activities of the individual species-like populations whose ensemble activities comprise guild activities. Our ultimate goal is to understand the unique functional contributions of each species-like population in a microbial community. Here, evolutionary ecology may suggest a new way of proceeding. As mentioned above (Fig. 8B) when distinct ecological populations diverge, all genes should be subject to neutral mutational (including recombinational) changes that lead to distinctive allelic variants in each ecological population. The distinction of allelic variants in different ecological populations may provide a means of analyzing ecotype-specific gene expression. Emerging results (Steunou et al. 2006) demonstrate the feasibility of *in situ* gene expression studies in our mat system and we are aiming at ecotype-specific gene expression studies in future work. This would put us in a position to evaluate how distinct ecotype populations express genes spatiotemporally in the community.

It should be clear that Dutch scientists, many associated with the Delft School of Microbiology, have had a significant impact on research in my laboratory, and, for this I am very grateful. I look forward to continued collaborations. I have often wondered how Martinus Beijerinck, the first head of the Delft School of Microbiology, would have reacted to the kind of research we have done together. Gijs Kuenen, the retiring head of the Delft School of Microbiology, thought he would have enjoyed it, as evidenced by his comments in a copy of Verzamelde Geschriften van M.W. Beijerinck (van Iterson et al. 1940) he presented to my lab after his visit in 1991.

When Beijerinck retired, he said, ''Gelukkig zij die nu beginnen'': ''Happy those who are beginning now.'' Clearly, at that time (1921) Beijerinck could see the tremendous new possibilities for new research which had come up and was envying the youngsters, who were just starting off, when he was forced to retire. No doubt, today he would have said and felt the same, and I am sure that Beijerinck would have liked to do the kind of research you are doing here in Bozeman on DNA and RNA probing of creatures in Nature.

For me the benchwork in Bozeman has been both a happy beginning and returning to DNA and RNA work that I started when I majored in biochemistry 25 years ago. It also has opened new possibilities for future research in Delft.

Now, on the occasion of Gijs' retirement, this comment has relevance for Gijs himself. He must have considered himself a youngster in 1991 and indeed carried molecular methods into the department in Delft. Though retiring, I'm certain that he will continue to be young at heart, sharing his insight and wisdom long into the future. I'm looking forward to how his ideas influence my future science. I'm also looking forward to his continuing friendship, as he has led the Delft School of Microbiology by showing us how the combination of good science and good nature can be both beneficial and rewarding.

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References

Allewalt JA, Bateson MM, Revsbech NP, Slack K, Ward DM (2006) Temperature and light adaptations of *Synechococcus* isolates from the microbial mat community in Octopus Spring, Yellowstone National Park. Appl Environ Microbiol 72:544–550

Anderson KL, Tayne TA, Ward DM (1987) Formation and fate of fermentation products in hot spring cyanobacterial mats. Appl Environ Microbiol 53:2343–2352

Bateson MM, Ward DM. (1988) Photoexcretion and fate of glycolate in a hot spring cyanobacterial mat. Appl Environ Microbiol 54:1738–1743

Bauld J, Brock TD (1973) Ecological studies of *Chloroflexis*, a gliding photosynthetic bacterium. Arch Microbiol 92:267–284

Begon M, Harper JL, Townsend CR (1996) Ecology: individuals, populations and communities, 2nd edn. Blackwell Scientific Publications, Cambridge, MA

Brock TD (1978) Thermophilic microorganisms and life at high temperatures. Springer-Verlag, New York

Cohan FM (2002) What are bacterial species? Ann Rev Microbiol 56:457–487

de Bruyn J, Boogerd FC, Bos P, Kuenen JG (1990) Floating filters, a novel technique for isolation and enumeration of fastidious, acidophilic, iron-oxidizing, autotrophic bacteria. Appl Environ Microbiol 56:2891–2894

Ferris MJ, Muyzer G, Ward DM (1996a) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol 62:340–346

Ferris MJ, Ruff-Roberts AL, Kopczynski ED, Bateson MM, Ward DM (1996b) Enrichment culture and microscopy conceal diverse thermophilic *Synechococcus* populations in a single hot spring microbial mat habitat. Appl Environ Microbiol 62:1045–1050

Ferris MJ, Ward DM (1997) Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl Environ Microbiol 63:1375–1381

Ferris MJ, Kühl M, Wieland A, Ward DM (2003) Different light-adapted ecotypes in a 68°C *Synechococcus* mat community revealed by analysis of 16S-23S intervening transcribed spacer variation. Appl Environ Microbiol 69:2893–2898

Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E., van de Peer Y, Vandamme P, Thompson FL, Swings J (2005) Reevaluating prokaryotic species. Nature Rev Microbiol 3:1–7

Goodfellow M, Manfio GP, Chun J (1997) Towards a practical species concept for cultivable bacteria. In: Claridge MF, Dawah HA, Wison MR (eds) Species: the units of biodiversity. Chapman and Hall, London, pp 25–59

Hanada S, Takaichi S, Matsuura K, Nakamura K (2002) *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium which lacks chlorosomes. Int J Syst Evol Microbiol 52:187–193

Herter S, Fuchs G, Bacher A, Eisenreich W (2002) A bicyclic autotrophic CO_2 fixation pathway in *Chloroflexus aurantiacus*. J Biol Chem 277:20277–20283

Holo H, Sirevåg R (1986) Autotrophic growth and CO_2 fixation of *Chloroflexus aurantiacus*. Arch Microbiol 145:173–180

Hutchinson GE (1957) Concluding remarks. Cold Spring Harbor Symp Quant Biol 22:415–427

Kallas T, Castenholz RW (1982) Rapid transient growth at low pH in the cyanobacterium *Synechococcus* sp. J Bacteriol 149:237–246

Mayr E (1982) The growth of biological thought: diversity, evolution and inheritance. Harvard Univ. Press, Cambridge, MA

Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 95:3140–3145

Meeks JC, Castenholz RW (1971) Growth and photosynthesis in an extreme thermophile, *Synechococcus lividus* (cyanophyta). Arch Mikrobiol 78:25–41

Miller SR, Castenholz RW (2000) Evolution of thermotolerance in hot spring cyanobacteria of the genus *Synechococcus*. Appl Environ Microbiol 66:4222–4229

Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nold SC, Ward DM (1996) Photosynthate partitioning and fermentation in hot spring microbial mat communities. Appl Environ Microbiol 62:4598–4607

Nübel U, Bateson MM, Vandieken V, Wieland A, Kühl M, Ward DM (2002) Microscopic examination of distribution and phenotypic properties of phylogenetically diverse *Chloroflexaceae*-related bacteria in hot spring microbial mats. Appl Environ Microbiol 68:4593–4603

Nübel U, Garcia-Pichel F, Clavero E, Muyzer G (2000) Matching molecular diversity and ecophysiology of benthic cyanobacteria and diatoms in communities along a salinity gradient. Environ Microbiol 2:217–226

Pierson BK, Castenholz RW (1974) A phototrophic gliding filamentous bacterium of hot springs, *Chloroflexus aurantiacus*, gen. and sp. nov. Arch Microbiol 100:5–24

Ramsing NB, Ferris MJ, Ward DM (2000) Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter thick photic zone of a hot spring cyanobacterial mat Appl Environ Microbiol 66:1038–1049

Revsbech NP, Ward DM (1984) Microelectrode studies of interstitial water chemistry and photosynthetic activity in a hot spring microbial mat. Appl Environ Microbiol 48:270–275

Rocap G, Larimer FW, Lamerdin J, Malfatti S, Chain P, Ahlgren NA, Arellano A, Coleman M, Hauser L, Hess WR, Johnson ZI, Land M, Lindell D, Post AF, Regala W, Shah M, Shaw SL, Steglich C, Sullivan MB, Ting CS, Tolonen A, Webb EA, Zinser ER, Chisholm SW (2003) Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. Nature 424:1042–1047

Ruff-Roberts AL, Kuenen JG, Ward DM (1994) Distribution of cultivated and uncultivated cyanobacteria and *Chloroflexus*-like bacteria in hot spring microbial mats. Appl Environ Microbiol 60:697–704

Sandbeck KA, Ward DM (1981) Fate of immediate methane precursors in low sulfate hot spring algal-bacterial mats. Appl Environ Microbiol 41:775–782

Santegoeds CM, Nold SC, Ward DM (1996) Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. Appl Environ Microbiol 62:3922–3928

Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849

Stewart WDP (1970) Nitrogen fixation by blue-green algae in Yellowstone thermal areas. Phycologia 9:261–268

Steunou A-S, Bhaya D, Bateson M, Melendrez M, Ward DM, Brecht E, Peters JW, Kühl M, Grossman A (2006) *In Situ* analysis of nitrogen fixation and metabolic switching in unicellular thermophilic cyanobacteria inhabiting hot spring microbial mats. Proc Natl Acad Sci USA 103:2398–2403

van der Meer MTJ, Schouten S, Ward DM, Geenevasen JAJ, Sinninghe Damsté JS (1999) All-cis hentriaconta-9,15,22-triene in microbial mats formed by the phototrophic prokaryote *Chloroflexus*. Org Geochem 30:1585–1587

van der Meer MTJ, Schouten S, de Leeuw JW, Ward DM (2000) Autotrophy of green non-sulphur bacteria in hot spring microbial mats: biological explanations for isotopically heavy organic carbon in the geological record. Environ Microbiol 2:428–435

van der Meer MTJ, Schouten S, Hanada S, Hopmans EC, Sinninghe Damsté JS, Ward DM (2002) Alkane-1,2-diol-based glycosides and fatty glycosides and wax esters in *Roseiflexus castenholzii* and hot spring microbial mats. Arch Microbiol 178:229–237

van der Meer MTJ, Schouten S, Sinninghe Damsté JS, de Leeuw JW, Ward DM (2003) Compound specific isotopic fractionation patterns suggest different carbon metabolisms among *Chloroflexus*-like bacteria in hot spring microbial mats. Appl Environ Microbiol 69:6000–6006

van der Meer MTJ, Schouten S, van Dongen BE, Rijpstra WIC, Fuchs G, Sinninghe Damsté JS, de Leeuw JW, Ward DM (2001) Biosynthetic controls on the ^{13}C-contents of organic components in the photoautotrophic bacterium *Chloroflexus aurantiacus*. J Biol Chem 276:10971–10976

van der Meer MTJ, Schouten S., Bateson MM, Nübel U, Wieland A, Kühl M, De Leeuw JW, Sinninghe Damsté JS, Ward DM (2005) Diel variations in carbon metabolisms of green nonsulfur-like bacteria in alkaline silicious hot spring microbial mats from Yellowstone National Park, USA. Appl Env Microbiol 71:3978–3986

van Dongen BE, Schouten S, Sinninghe Damsté JS (2001) Gas chromatography/combustion/isotope-ratio-monitoring mass spectrometric analysis of methylboronic derivatives of monosaccharides: a new method for determining natural ^{13}C abundances of carbohydrates. Rapid Commun Mass Spectrom 15:496–500

van Iterson G Jr, den Dooren de Jong LE, Kluyver AJ (1940) Verzamelde Geschriften van MW Beijerinck. Zesde Deel, Martinus Nijhoff, s'Granenhage, NL

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers Y-H, Smith HO (2004) Environmental shotgun sequencing of the Sargasso Sea. Science 304:66–74

Ward DM (1998) A natural species concept for prokaryotes. Curr Opin Microbiol 1:271–277

Ward DM (2006) A macrobiological perspective on microbial species. Microbe (in press)

Ward DM, Bateson MM, Ferris MJ, Nold SC (1998) A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol Mol Biol Rev 62:1353–1370

Ward DM, Bateson MM, Weller R, Ruff-Roberts AL (1992) Ribosomal RNA analysis of microorganisms as they occur in nature. Adv Microbial Ecol 12:219–286

Ward DM, Castenholz RW (2000) Cyanobacteria in geothermal habitats. In: Potts M, Whitton B (eds) Ecology of Cyanobacteria. Kluwer Academic Publishers, The Netherlands, pp 37–59

Ward DM, Cohan FM (2005) Microbial diversity in hot spring cyanobacterial mats: pattern and prediction.In: Inskeep WP, McDermott TR (eds) Geothermal biology and geochemistry in Yellowstone National Park. Montana State University Thermal Biology Inst., Bozeman, MT, pp 185–202

Ward DM, Papke RT, Nübel U, McKitrick MC (2002) Natural history of microorganisms inhabiting hot spring microbial mat communities: clues to the origin of microbial diversity and implications for micro- and macro-biology. In: Staley JT, Reysenbach A-L (eds) Biodiversity of microbial life: foundation of earth's biosphere. John Wiley and Sons, NY, pp 25–48

Ward DM, Santegoeds CM, Nold SC, Ramsing NB, Ferris MJ, Bateson MM (1997) Biodiversity within hot spring microbial mat communities: molecular monitoring of enrichment cultures. Antonie van Leeuwenhoek 71:143–150

Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 344:63–65

Wickstrom CE (1980) Distribution and physiological determinants of blue-breen algal nitrogen fixation along a thermogradient. J Phycol 16:436–443