ADAPTATIONS OF THE BACTERIAL FLYWHEEL FOR OPTIMAL MINERAL CYCLING IN OLIGOTROPHIC SURFACE WATERS

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Abstract

Nutrient cycling in a subarctic oligotrophic lake was explored using current kinetic theory for organisms adapted to low nutrient environments with emphasis on bacterial contributions to system function. Techniques were refined which minimize sample disturbance and contamination for the purpose of accurately measuring bacterioplankton activity. Seasonal variations in DNA content, cell mass, species composition, specific affinity for amino acids and cell yield were observed. Quasi-steady state formulae describe bacteria as a flywheel in nutrient cycling; energy is conserved within a relatively constant biomass by varying bacterial activity with nutrient availability.

The bacterial flywheel paradigm provides a bacteriocentric view of mineral cycling, linking kinetics to specific cytoarchitectural properties while maintaining links to substrate and grazing pressures. As an extention of the microbial loop paradigm, the flywheel becomes essential at high latitudes. In winter, low solar input interrupts the microbial loop so that the dissolved organic carbon (DOC) pool is cycled through bacteria only. This activity allows bacterioplankton to persist through winter and respond rapidly to springtime warming and nutrients.

Microbial adaptations to seasonal variations in nutrient availability and temperatures were examined within the bacterial flywheel framework. Organisms are well-adapted to a narrow (17 °C) in situ temperature range. Activation energies for small warming were low at the temperature extremes (20.6 kJ mol^{-1} at 0.5 °C; -32 kJ mol^{-1} at 17 °C) and high in spring (110 kJ mol^{-1} at 1.2 °C). Nutrition varies by season, supplied in large part by amino acids in spring and summer. Winter growth rates are at least 0.013 day^{-1} whereas partial growth rate on amino acids for that season is only 2.8×10^{-5} day^{-1}. It is proposed that winter organisms rely on diffusion transport and/or shift toward concurrent use of a large suite of substrate types for growth and maintenance.

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1 Introduction

Quantifying bacterial activity is important to understanding their role in mineral cycling. However difficulties arise due to the concentrations of substrate which are small and of diverse structure, cell mass that is of insufficient size for measurement by conventional methods, slow nutrient flux, and the failure of most species to reproduce as isolated cultivars. Additionally, uncertainties exist in values for in situ cell yield and in the temperature-dependent changes in metabolic activity. These uncertainties are due in part to measurement difficulties, particularly at remote sites with limited equipment, and to the use of kinetic theory borrowed from early studies of isolated enzymes in defined systems, which disregards changes in the physiological properties resulting from sustained steady-state growth in a nutritionally complex mixture. Radiotracers with high specific activity may be used to measure uptake, but only a limited number of substrate types are known (Andrews and Williams 1971; Carlucci et al. 1984; Hollibaugh and Azam 1983; Kirchman et al. 2001; Pakulski and Benner 1994; Rich et al. 1996) and short incubation times are required to limit bottle effects (Ferguson et al. 1984; Ferguson and Sunda 1984; Šimek et al. 2005). Microbial use of very small concentrations of complex molecules may be too slow to accurately resolve activity by uptake studies even if radiotracers become available. Activity is best normalized to biomass but since these organisms are small, measurements require very high resolution techniques (Robertson et al. 1998). Published estimates of yield vary widely by experimental conditions as well (Jahnke and Craven 1995). Many of these problems would be alleviated by cultivation of typical organisms but few have been successfully isolated (Button et al. 1998; Connon and Giovannoni 2002; Kogure et al. 1979). Consequently, bacteria are often assigned a 'black box' within food web models (Barber 2007; Thingstad 2000) and details of their function are deduced from fluxes between other boxes in the model.

Traditionally, bacteria have been regarded primarily as remineralizers, converting minerals in organic matter to their inorganic form to be used again by phototrophs in surface water. Initial estimates of bacterial biomass were low, leading to the conclusions that bacteria were relatively unimportant to mineral cycling in surface water and that

bacteria, zooplankton and fish in the aphotic zone were the principle remineralizers. Then, the 'microbial loop' concept was introduced to account for carbon flux through bacterioplankton to higher trophic levels (Azam et al. 1983; Williams 1995). In this model, some portion of the dissolved organic matter released from phytoplankton and higher trophic level organisms is used by bacteria, returning material and energy to higher trophic levels through grazers of bacteria, namely heterotrophic microflagellates and microzooplankton. New methods of bacterial enumeration (Bowden 1977; Hobbie et al. 1977) and activity (Hagström et al. 1979; Kogure et al. 1979) supported the expanding role of bacteria in mineral cycling models.

In this study seasonal data for a sub-arctic lake are used to construct a model, the bacterial flywheel, which describes links from bacterial activity to both higher trophic level biomass and to carbon dioxide production. Data are consistent with the concept that bacteria operate as a flywheel in mineral cycling. Throughout the annual cycle, bacterial biomass remains relatively constant. In winter, when the microbial loop is interrupted by the absence of solar energy input, bacteria maintain their capacity to take up organic substrate and continue to grow. This results in springtime bacterial populations which are sufficiently abundant and active to respond readily to warmer conditions. Three water types from the annual cycle and their properties are described within this context.

The model also provides a framework for examining specific adaptations by bacterioplankton communities to seasonal changes in their environment. Application of novel methods allowed the quantification of microbial activity toward particular nutrients in the presence of others suitable for use in natural waters. These methods included kinetic theory, flow cytometry, extinction culture, and radioisotope techniques. Radioisotope techniques employed for the purpose of kinetic and metabolic efficiency considerations in particular were developed to optimize precision and address common problems in studying bacterioplankton.

Radiotracers added at the time of sample collection have been used to obtain estimates of naturally occurring nutrient concentrations and turnover times according to a Wright-Hobbie analysis of the departure from hyperbolic kinetics; however, the analysis is

imprecise (Logan and Fleury 1993). An alternative strategy, employed here, is based on specific affinity theory (Button et al. 1973). Kinetic considerations (Button et al. 2004) indicated that the pseudo first-order rate constant (specific affinity) of a substrate can be determined from radiotracer uptake if permeases do not discriminate between labeled and unlabeled substrate. Total uptake rate of the chemical species may be determined as well within the accuracy that the ambient concentration of the tracer substrate is known. Further nutrient flux could be evaluated with the accuracy that ambient nutrient concentrations could be measured, leading to increased understanding of the annual microbial cycle. To circumvent difficulties inherent in quantitative nutrient chemistry, in situ specific affinities were used here to determine minimum nutrient fluxes, and to indicate seasonal temperature response.

Radiotracers were also used in short term incubations to ascertain values for metabolic efficiency. Values for oligobateria are uncertain (del Giorgio et al. 1997) due to difficulties in establishing conversion factors and verifying assumptions. However, cell yields obtained from radiolabel conversion are more direct. Values obtained for Harding Lake with this method and reported here are closer to those established for more easily manipulated cultivars (Johnson 1967).

Temperature, a major seasonal factor in the sub-arctic, was examined for effects on metabolic activity. The temperature-dependency of the rates of biological reactions such as enzymatic conversions, typically double with a temperature rise of 10°C. However, we (Button et al. 2004) and others (Yager and Deming 1999) found much stronger effects in the North Pacific, as compared to most studies (see Price and Sowers (2004) for review), while Georlette et al. (2003) provided biophysical insight. In Harding Lake we had observed changes in the temperature effect with all samples adjusted to 10°C to minimize variables (unpublished). Here rates were measured at ambient temperatures along with rates in parallel samples following temperature manipulation. It confirmed results of the previous work showing strong seasonal variation, and in addition demonstrated changes in organism physiology. Moreover, resulting profiles in apparent activation energy along with the specific affinity data support the concept of a bacterial flywheel.

Previous success with extinction cultures demonstrated an improvement in isolation success from about 0.1% to 2% by incubation in raw seawater alone (Button et al. 1993). I wondered if success could be further improved by more closely duplicating in situ chemistry. This was attempted, with extension into a freshwater system, by supplementing sterilized lakewater with lyophil, or by partitioning autoclaved lakewater across membrane filters against raw lakewater. While the procedures worked, improvements using more permeable membrane filters gave only limited success due to failures of the membrane filters. Equations had been derived to account for this problem (Quang and Button 1998), but uncertainties were excessive.

The bioenergetic basis of the bacterial flywheel, from the second law of thermodynamics, depends on energy from dissipative processes such as heterotrophic metabolism. Molecular organization is attained at the expense of Helmholtz free energy during flow toward a reduced entropic state, flow that is decreased principally through configurational and thermal randomness (Wicken 1979). Functional structures such as enzymes contain the same energy as nonfunctional ones, but are favored in open systems by self replication. Thus, while formation of functional structures is rare over short periods, they persist leading to accumulation and system development. A property of the evolving functional and replicative complexions is the tendency to maximize biomass from available resources. In phytoplankton part of the replicative process often includes spores to seed springtime blooms (Eilertsen et al. 1995). Aquatic bacteria, usually Gram negative and non-spore forming, appear to assume a state of minimal energy consumption to bridge an annual gap of low photosynthate production. Bioenergetic costs can be minimized because little cytochemical restructuring is required to resume growth in springtime, and costs of active transport may be avoided by reliance on diffusion for nutrient transport in cold, low-energy environments. The result, proposed here, is an extended period for mineral-rich primary productivity in the short summers of the far north.

2 Materials and Methods

2.1 Study site and sampling.

Harding Lake is a mostly dimictic lake located in interior Alaska (64°25'N, 146°50'W). It is deep (maximum, 43 m) compared to most interior lakes with an aerial extent of 988 ha (Blackwell 1965). It is adjacent to a state recreation site and private homes and is stocked periodically for recreational fishing. The lake is considered oligotrophic with average summer chlorophyll *a* concentrations in the photic zone ranging from ~1 to 1.7 μg L^{-1} (Gregory-Eaves et al. 2000; LaPerriere et al. 2003) and dissolved organic carbon concentrations ≤ 5 mg L^{-1} (LaPerriere 2003). A history of research on the lake, prompted by local residents' concern regarding a receding water level, is summarized by LaPerriere (2003).

Sampling was conducted from float plane or canoe. All glassware was combusted at 550°C for two hours. Glass carboys were filled with surface water by glass carafe or with lakewater collected 1 m under the ice using glass tubing and a hand pump. Holes in the ice were chiseled with a clean ice chipper. Samples were transported to the lab in insulated boxes. Incubation and culturing experiments were begun within two hours of sample collection. Sample preparation and incubations were performed in walk-in incubators under ambient light except where noted.

Flow cytometry, HPLC and respiration rate measurements were conducted by Betsy Robertson in 1990 and 1991. During the period of 2001 through 2004, flow cytometry analyses were conducted by Garrett Pernéy, myself and Doug Macintosh. Incorporation rate measurements and bottle effect experiments were conducted by me. Yield and culture data were also generated by me except where noted. TRFLP was conducted by Zhao (2005). Yield, temperature, bottle effect and genomic work were conducted from 2001 through 2003. Depth profiles were generated from samples collected by Niskin bottle in 2003 and formaldehyde-preserved on site.

2.2 Flow cytometry and high performance liquid chromatography (HPLC).

Bacterial biomass, population and DNA content of preserved (0.5% formaldehyde) 1.0 μm filtrate were measured by flow cytometry (Button and Robertson 2001; Robertson et

al. 1998). A conversion factor for dry weight to wet weight of 0.18 was used for cell mass calculations (Button and Robertson 2000). Cytograms such as those in Fig. 1 were examined for DNA distributions. The abundance at the mode DAPI-DNA value was compared to the abundance at four times that mode and the percentage calculated. Cell mass was treated similarly. Cytograms of in situ samples often displayed bimodal distributions similar to cultures in log phase. Here, organism size was presented as a surrogate for growth rate because large organisms are more robust and contain larger amounts of RNA and enzymes, and because replication rate can exceed division rate (Robertson and Button 1989). Samples containing a high percentage of cells with the higher DNA content (4 times the mode) were presumed to have a higher percentage of replicating cells.

The following amino acids were quantified by HPLC: aspartic acid, glutamic acid, serine, glycine, alanine, leucine and lysine. Up to 3 mL samples were collected, transported cold, filtered through 0.2 μm Millipore filters (previously rinsed with sample) and frozen until assayed. HPLC analysis was with a Spectra-Physics 8700 solvent delivery system, Whatman PRP-1 column, Kratos FS950 fluoromat detector, Spectra-Physics 4270 integrator, and a Gilson 201 fraction collector according to previously reported methods (Carlucci et al. 1984). Total amino acids were calculated by assuming that aspartic acid, serine, glycine and alanine comprise 74% of the total (Button and Robertson 1989).

2.3 Bacterial activity.

Respiration rates were measured during 1990 and 1991 (unpublished). Four liters of sample were filtered through 1.0 μm Nuclepore filters, brought to 10°C and incubated with 0.2 μg L^{-1} of [^{14}C(U)]-L-amino acid mixture (NEC-445, DuPont). $^{14}CO_2$ was collected from 0.4 L of acidified subsamples as for yield experiments, four times over the course of two to four hours (n=1). The slope of the regression line was calculated from the $^{14}CO_2$ evolution curve and was taken as the respiration rate, v_L, of radiolabeled compounds.

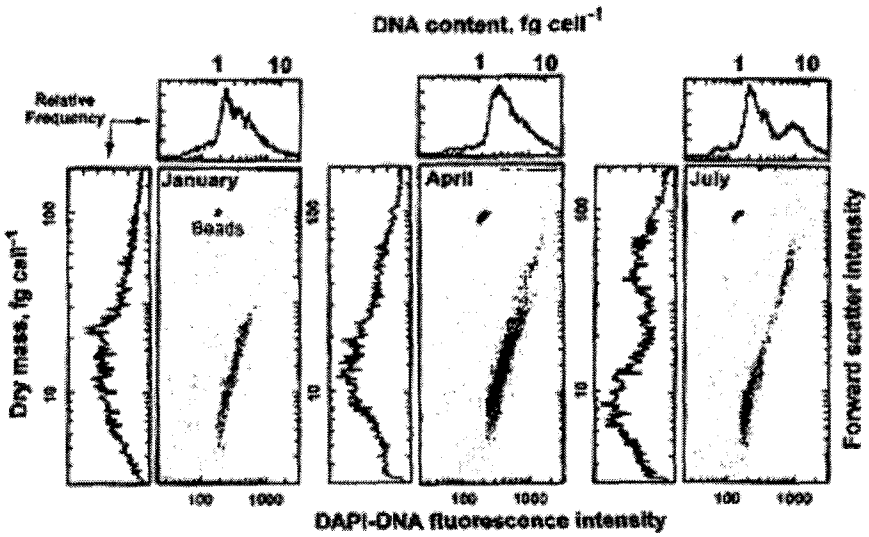

Figure 1. Flow cytometry cytograms of Harding Lake surface water. DNA and biomass distributions were different in Jan, Apr and Jul.

Specific affinities, a_S^0, for amino acids were calculated from the respiration rates:

$$\sum_{i=1}^{n} a_{S_i}^0 = \sum_{i=1}^{n} \frac{v_L}{S_i \; Y_R \; X}. \qquad (1)$$

where Y_R is the ratio of substrate respired to substrate utilized:

$$Y_R = \frac{CO_2}{X + CO_2 + \text{products}}. \qquad (2)$$

S is the concentration of labeled substrate and X is the wet weight of the biomass determined by flow cytometry. For simplicity a yield of 0.5 was used for all specific affinities.

Respiration, or incorporation, of background substrate (v_B) occurs simultaneously so that metabolism of all substrates ($S_L + S_B$) is:

$$v_L + v_B = \frac{dX_L}{dt} + \frac{dX}{dt} = v_T. \qquad (3)$$

Assuming cells cannot differentiate between labeled and unlabeled substrate and added substrate concentrations are subsaturating, incorporation of each substrate occurs at the same rate as that of its labeled counterpart. If background concentrations are known, specific affinities derived from radiolabel measurements may be used to determine total rates.

2.4 Uptake rates and bottle effect.

The effect of sample volume, filtration and bottle pre-treatment on uptake rates was determined by incubation of multiple parallel samples with a mixture of thirteen [^{3}H]-L-amino acids (ART-328, ARC). All bottle effect experiments were performed at in situ temperatures. Ethanol was evaporated from the amino acid mixture overnight and the mixture was reconstituted with sterile glass distilled water and filtered with a 0.2 μm syringe filter prior to use. Samples were assayed for radiolabel incorporation at least four times within five hours. For each time point 10 mL of sample were removed and immediately filtered onto 0.2 μm polycarbonate membrane filters (Millipore GTTP02500). Filters were rinsed three times with glass distilled water and inserted into ReadySafe scintillation fluid (BeckmanCoulter 141349). One hundred μl were taken from

each bottle for a total count. Radioactivity incorporated into filterable material was measured with a scintillation counter. Incorporation rates were calculated from the initial linear range of the uptake curve. For four hour incubations, the coefficient of determination (r^2) values were always greater than 0.970 and often greater than 0.990.

To determine the effect of incubation volume on uptake rates, 6 100-mL and 6 1-L samples were incubated in 200-mL and 2-L bottles respectively, and incubated with 5.6 x 10^6 DPM L^{-1} (0.14 µg amino acids L^{-1}). Due to small sample sizes, the Mann-Whitney statistical test was chosen to compare mean incorporation rates (% h^{-1}) between the two volumes used.

The filtration effect of GF/D glass fiber filters (2.7 µm retention size) was examined in two identical filtration systems. Three samples were taken from the filtrate of each system and incubated with 3.3 x 10^7 DPM L^{-1} (0.082 µg amino acids L^{-1}). Six unfiltered samples were incubated in parallel. For the pre-treatment experiment, three 200-mL glass bottles each were pre-soaked for one day in lakewater, 10^{-6} M EDTA or 10 N HNO_3. Three bottles were not pre-treated. Bottles were rinsed thoroughly with glass distilled water, checked with a pH meter to ensure complete rinsing, and then rinsed with fresh lakewater prior to filling. One hundred mL lakewater were measured into each bottle and incubated with 9.0 x 10^6 DPM L^{-1} (0.023 µg amino acids L^{-1}). Due to small sample size, the Kruskal Wallis test was performed on the incorporation rates (% h^{-1}) to determine a difference in any one mean incorporation rate among the four treatment sets.

2.5 Yield.

The ^{14}C-amino acid mixture (L-[U-^{14}C]amino acid mixture, Amersham Biosciences CFB104, 2% ethanol; s.a. 1.92 GBq/milliatom: 52.0 mCi/milliatom) was dried overnight in a vacuum chamber, reconstituted with sterile glass distilled water and filtered with a 0.2 µm PVDF syringe filter (Millipore, Acrodisc LC 13 mm) just prior to use. ^{14}C-glucose (Glucose, D-[^{14}C(U)], ARC-122D, 0.1 mCi/mL sterile water, s.a. 4mCi/mmol, 50µCi/1.85MBq) was diluted in sterile glass distilled water. Twenty L volumes were collected for each experiment. One L subsamples were incubated with ^{14}C-amino acids or ^{14}C-glucose in duplicate to final concentrations of 0.1 µg L^{-1} and 1.0 µg L^{-1} respectively.

One hundred µl from each bottle was analyzed for total radioactivity. At regular intervals (3 times within 7 hours), 20 mL from each were acidified with 1 N H_2SO_4 to a pH between 3.4-3.5 and sparged with N_2 at 0.5 L min^{-1}. For CO_2 collection scintillation vial caps were fitted with glass tubing, the vials filled with phenethylamine scintillation cocktail (PEA) and set three in a series to test for completeness of retrieval. PEA was prepared within two months of use as follows: 5.103g Omnifluor (Perkin Elmer 6NE9063); 270mL methanol, anhydrous (Mallinckrodt 3004-08); 461mL toluene (Baker, 1-9460); 270mL phenethylamine, 99% (Aldrich 128945). The particulate fraction was captured from 10 mL of incubation sample on 0.2 µm polycarbonate filters and rinsed well with glass distilled water. Filters were placed in PEA for counting. $^{14}CO_2$ collection efficiency of this system was evaluated periodically by examining bicarbonate radioactivity acid-released from pure water.

To determine the photosynthetic contribution to $^{14}CO_2$ flux in our samples, one experiment included incubation under ambient light (~ 7 µmol photons m^{-2} s^{-1}) and one in the dark (n=2). A difference in rates between light and dark incubations would indicate a measurable contribution of photosynthetic activity to ^{14}C allocation. All other incubations were conducted under ambient light. Results were analyzed for a significant difference in means with the Mann-Whitney test.

Yield was calculated as the ratio of cell material accumulation rate to uptake rate for incubations with either ^{14}C-glucose or ^{14}C-amino acids. These rates were calculated from the slope of the regression curve for radiolabel accumulation over 6 hours. To detect isotope dilution effects, yield was also calculated as the percent of cell material formed from total substrate taken up by the cells for each time point [($g_{cell\text{-}dry}$ $g_{amino\text{-}acids\text{-}dry}^{-1}$) x 100%)].

2.6 Extinction cultures.

Dilute inocula were cultured in either glass tubes or diffusion chambers (Fig. 2). Care was taken to minimize sample disturbance and to eliminate exogenous substrate and inhibitors to growth. Cultures were continuously illuminated at 15 µmol photons m^{-2} s^{-1}. Inocula sizes, substrate amendments and resulting viability estimates are reported in

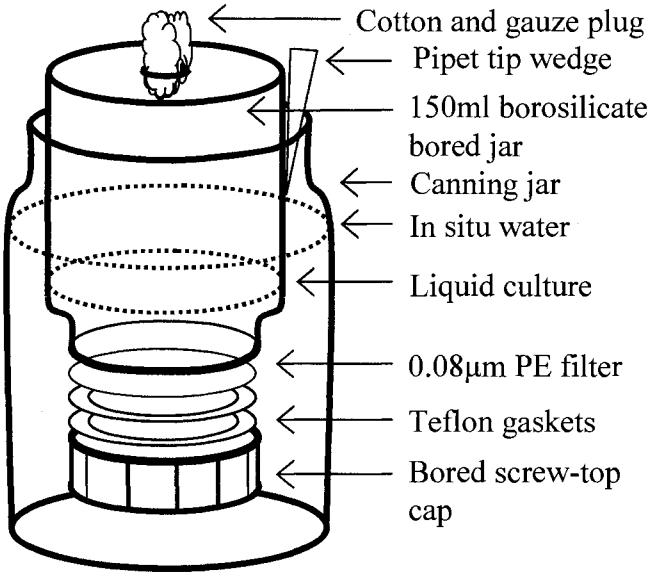

Figure 2. Diagram of a diffusion culture. The culture jar was assembled, then the entire set-up autoclaved. The reservoir was filled with in situ water; then the jar was allowed to rest on the bottom of the reservoir. Media entered via the 0.08μm filter overnight to a volume of approximately 20 mL. Inoculation was through the topmost port.

Table 1. Lyophil was obtained from surface water collected on 22 Aug 2001 and filtered through Whatman GF/D and 0.2 μm membrane filters at in situ temperature. Filtration was commenced within two hours after collection. Water was chilled less than one day prior to lyophilization. For culture amendment, lyophil was rehydrated with filtered autoclaved lakewater (FAL) and autoclaved. The volume of the lyophil was brought up with FAL for a final concentration of organics equal to six times that of the original water. Sodium acetate (Mallinckrodt) was dissolved in glass distilled water to a concentration of 100 mg L^{-1}, then autoclaved in sealed ampules. α-D(+)glucose (Sigma) was dissolved to a concentration of 100 mg L^{-1} and stored similarly. The following amino acids were obtained individually from Sigma in powder form and dissolved together in glass distilled water at a total concentration of 10 g amino acids L^{-1}. Their proportions are similar to the amino acids found in *Chlorella sp.* (Sorimachi 1999) and are indicated in parentheses: aspartic acid (0.090), glycine (0.049), alanine (0.073), arginine (0.073), cysteine (0.0026), glutamic acid (0.122), histidine (0.018), isoleucine (0.049), leucine (0.085), lysine (0.079), methionine (0.022), phenylalanine (0.049), proline (0.071), serine (0.037), threonine (0.037), tryptophan (0.023), tyrosine (0.054) and valine (0.068). Stock solutions were further diluted in sterile glass distilled water just before addition to cultures.

Inoculum size was initially determined by epifluorescence microscopy and later confirmed by flow cytometry with preserved samples. Incubation times were three weeks for summer and five weeks for winter experiments. Sub-samples were preserved in 0.5% formalin and stained with DAPI and analyzed by flow cytometry as previously described. Cultures were scored positive for growth by flow cytometry when detectable populations of about 10^4 organisms appeared in the forward scatter and apparent DNA cytograms. Three mL of DAPI-stained sub-samples were filtered for microscopy. Twenty fields or 100 cells were viewed per filter. Cultures were scored positive by this method if cell concentrations appeared $\geq 10^4$ cells mL^{-1} and fluorescing particles were distinguishable from a blank. The blanks for each session were prepared as for the cultures but contained filtered autoclaved lakewater (FAL).

Table 1. Harding Lake dilution culture experiments.

Experiment Date	System	Culture vessel, incubation temperature	Culture media	Substrate amendment	Inoculum size, experiments, positive cultures (species)[1]	Viability estimate (%), se (%)[2]
10 Aug 2001	Limnic	Glass tubes, 16°C	FAL	None	438, 10, 6(10)	0.23, 0.12
					44, 10, 5(9)	1.9, 2.7
					0, 14, 0 ***Control***	
					All experiments	0.21, 0.10
				Periodic addition[3]	438, 10, 9(11)	0.25, 0.12
					44, 10, 3(4)	0.85, 2.6
					0, 10, 2(2) ***Control***	
					All experiments	0.20, 0.09
				100 µg glucose L^{-1}	438, 10, 8(21)	0.48, 0.14
					44, 10, 1(5)	1.1, 2.6
					0, 10, 0 ***Control***	
					All experiments	0.55, 0.11
				100 µg mixed amino acids L^{-1}	438, 10, 4(13)	0.30, 0.12
					44, 10, 6(13)	2.8, 2.7
					0, 10, 1(1) ***Control***	
					All experiments	0.19, 0.11

[1] Inoculum size = average number of cells as determined by FCM population determination of the original sample and by its dilution; experiments = number of vessels inoculated; positive cultures were determined as described in the text; species = total number of species counted in all culture vessels in the group.

[2] Calculated with the statistical program Viabcal using S-Plus software as in reference Quang and Button (1998).

[3] Additions were as follows: Day_8, 1.0 µg amino acid mixture + 0.083 µg glucose L^{-1}; Day_{15}, 2.0 µg amino acid mixture + 0.17 µg glucose L^{-1}; Day_{22}, 4.0 µg amino acid mixture + 0.33 µg glucose L^{-1}; Day_{31}, 8.0 µg amino acid mixture + 0.67 µg glucose L^{-1}; Day_{39}, 20 µg amino acid mixture + 1.25 µg glucose L^{-1}.

Table 1 continued.

Experiment Date	System	Culture vessel, incubation temperature	Culture media	Substrate amendment	Inoculum size, experiments, positive cultures (species)[1]	Viability estimate (%), se (%)[2]
				100 µg glucose + 100 µg mixed amino acids L^{-1}	438, 10, 4(15)	0.34, 0.13
					44, 10, 0	0, 0
					0, 10, 0 ***Control***	
					All experiments	0.31, 0.08
19 May 2003	Limnic	Glass tubes, 5°C	FAL	None	66, 12, 2(17)	2.1, 1.4
					6.6, 12, 0	0, 0
					0.66, 12, 0	0, 0
					0, 12, 0 ***Control***	
					All experiments	2.0, 0.47
				Lakewater lyophil[4]	66, 3, 2(11)	5.4, 3.1
					6.6, 3, 2(14)	17, 93
					0.66, 3, 0	0, 0
					0, 3, 0 ***Control***	
					All experiments	5.6, 2.4
				100 mg acetate L^{-1}	66, 12, 1(37)	4.5, 1.5
					6.6, 12, 5(17)	12, 33
					0.66, 12, 1(6)	3.5, 230
					0, 12, 0 ***Control***	
					All experiments	4.7, 0.91
				100 µg mixed amino acids L^{-1}	66, 12, 6(30)	3.7, 1.5
					6.6, 12, 2(15)	9.9, 33
					0.66, 12, 1(4)	8.3, 308
					0, 12, 1(6) ***Control***	
					All experiments	3.2, 0.79

[4] 6x concentration.

Table 1 continued.

Experiment Date	System	Culture vessel, incubation temperature	Culture media	Substrate amendment	Inoculum size, experiments, positive cultures (species)[1]	Viability estimate (%), se (%)[2]
29 Sep 2004	Limnic	Diffusion chambers, 10°C	FL	None	2.1, 8, 2(6)	7.1, 114
					0.42, 8, 2(8)	13, na
					0, 8, 2(5) ***Control***	
					All experiments	0.01, na
				Periodic addition of glucose[5]	2.1, 8, 5(14)	3.6, 114
					0.42, 8, 2(5)	2.4, na
					0, 8, 1(2) ***Control***	
					All experiments	73, 25
				Periodic addition of amino acids[5]	2.1, 7, 4(12)	3.4, 128
					0.42, 8, 4(10)	15, na
					0, 7, 4(7) ***Control***	
					All experiments	33, 31
				Periodic addition of acetate[5]	2.1, 8, 0	0, 0
					0.42, 8, 4(17)	1.1, na
					0, 7, 1(1) ***Control***	
					All experiments	0, na
11 Oct 2004	Limnic	Glass tubes, 12°C	FAL	None	320, 10, 3(29)	0.91, 0.22
					120, 10, 3(22)	1.8, 0.70
					40, 10, 3(10)	2.3, 3.1
					3.2, 10, 1(3)	4.5, 72
					0, 10, 0 ***Control***	
					All experiments	1.1, 0.19

[5] Additions were as follows: Day_1, 10 pg substrate per culture; Day_{13}, 100 ng substrate per culture.

Table 1 continued.

Experiment Date	System	Culture vessel, incubation temperature	Culture media	Substrate amendment	Inoculum size, experiments, positive cultures (species)[1]	Viability estimate (%), se (%)[2]
				Periodic addition[6]	320, 10, 3(32) 120, 10, 3(24) 40, 10, 3(11) 3.2, 10, 2(3) 0, 10, 1(2) ***Control*** All experiments	1.0, 0.22 2.0, 0.73 2.6, 3.1 4.5, 72 1.2, 0.19

[6] Additions were a mixture of glucose and amino acids at the following concentrations: Day_1, 0.5 μg L^{-1}; Day_7, 1.25 μg L^{-1}; Day_{14}, 2.5 μg L^{-1}; Day_{21}, 5.0 μg L^{-1}.

Formulae from Quang and Button (1998) and their program Viabcal (S-PLUS) were used to calculate viability estimates and standard error for each treatment set. Input values were the number of species arising in each culture (including controls) detected by TRFLP (see section 2.8 and 2.9) assuming 100 species were present in the original water sample. This method carries these additional assumptions: 1) the number of cells inoculated into each culture vessel is random with the same known average value; 2) the level of contamination for each vessel is also random with the same unknown average value; 3) each cell introduced into a vessel has the same chance of coming from any of the species present in the original water sample; and 4) all cells have the same viability.

2.7 Temperature and exogenous substrate.

Our measurements were conducted over a wide seasonal temperature range. Surface water temperature was measured with a standard laboratory thermometer. Below ice temperature values were obtained with a YSI multiparameter meter. Surface water was warmed from in situ temperature in small 5-7°C increments and immediately incubated in parallel with ^{3}H-amino acids. Filterable radioactivity was measured similar to that in the bottle treatment experiments. The specific affinity was determined using a yield value of 0.5 g-cell g-substrate-utilized. The effect of warming was quantified as apparent activation energy:

$$E_a = \frac{R\left(\ln\frac{k_2}{k_1}\right)T_1T_2}{T_2 - T_1} \quad . \qquad (4)$$

k_1 and k_2 are the average specific affinities of replicate incubations at low and high temperatures respectively; T_1 and T_2 are the incubation temperatures of the lower (in situ) and higher (warmed) samples; and R is the gas constant. Some experiments included several steps in temperature. Incubations were performed at least in duplicate. Response of rate to temperature was presented as described by the traditional Arrhenius energy of activation. This construct normalizes change in the thermodynamic energy of activation to temperature for facile interpretation. Plots were constructed and analyzed for linearity and position relative to season.

To evaluate membrane potential depletion sub-samples were incubated with unlabeled glucose, 0 μg L^{-1} to 300 μg L^{-1}, in addition to the ^{3}H-amino acid mixture. Five experiments included glucose and were performed in March, April, May, July and September. The sample number in each treatment block was only two, so data could not be examined for normality and homoscedasticity. Consequently, 1-factor or 2-factor ANOVA was performed on the ranked specific affinity values from glucose and temperature experiments.

2.8 DNA extraction.

DNA was extracted from whole lakewater for terminal restriction length polymorphism (TRFLP) analysis in January, April and July. For each sample, 10 L of whole lakewater was filtered with GF/C filters (Whatman) to remove large particles and phytoplankton. Filtrate was concentrated onto 0.2 μm polyester filters (Poretics). Filters were cut into small pieces and stored in a microcentrifuge tube at -20°C. DNA extraction was with a DNeasy Tissue Kit (Qiagen). The sample was lysed in 200 μl of lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20 mg/mL lysozyme). Twenty μl of Proteinase K (20 mg/mL), 4 μl of RNase A (100 mg/mL), and 200 μl of Buffer AL were added to the sample. The sample was mixed immediately by vortexing, then incubated at 70°C for 10 minutes. Two hundred μl of 100% ethanol were added and the sample mixed thoroughly by vortexing. Sample DNA was bound to a DNeasy membrane by pipeting the sample onto a DNeasy mini column sitting in a 2-mL collection tube, then centrifuged at 8000 rpm (6000 x g) in an Eppendorf 5414 centrifuge for 1 minute. The mini column was placed in new 2-mL collection tubes, 500 μl of Buffer AW1 added and then centrifuged for 1 minute at 8000 rpm (6000 x g). The mini column was placed in new 2-mL collection tubes, 500 μl of Buffer AW2 added and then centrifuged for 3 minutes at 14,000 rpm (20,000 x g) to dry. To elute the DNA from the mini column, it was placed in a clean 1.5-mL microcentrifuge tube, 100 μl of Buffer AE placed directly on the membrane then incubated at room temperature for 1 minute. The mini column was centrifuged for 1 minute at 8000 rpm (6000 x g) followed by the addition of 100 μl of Buffer AE and incubation at room temperature for 1 minute. The

microcentrifuge tube was centrifuged for 1 minute at 8000 rpm (6000 x g) to collect the DNA filtrate. The extracted DNA samples were immediately frozen and stored at -20°C until further processing in the laboratory. Successful genomic DNA extraction was confirmed by running the filtrate on a 1.0% agarose gel. The gel was stained with SYBR Green I and photographed under a transilluminator.

DNA was also extracted from positive dilution cultures for TRFLP. Nine mL of each culture scored positive by flow cytometry and epifluorescence microscopy were put into Beckman Ultra-clear centrifuge tubes and pelleted in a Beckman L8 70M ultracentrifuge at 36,000 rpm (SW40 rotor) at 4°C for 60 minutes. The supernatants were discarded and the pellets were retained in the test tubes. The bacterial cells were lysed by freeze-thaw cycles (-20°C for 5 minutes, room temperature for 5 minutes, 5 cycles). The lysed cells were immediately frozen and stored at -20°C until further processing in the laboratory.

2.9 TRFLP.

16S rDNA from whole lakewater (three separate seasons) or individual cultures was amplified with forward primer (5'-GTTTGATCCTGGCTCAG-3') fluorescently labeled with 6-FAM (6-carboxyfluorescein) and backward primer (5'-ACGGTTACCTTG TTACGACTT-3') (Qiagen). PCR amplification began with a 2 minute denaturation at 95°C; this was followed by 30 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 1.5 minutes. The final cycle was extended at 72°C for 5 minutes. Purification was with Amicon Microcon-PCR Centrifugal Filter Devices (Millipore). Purified DNA was digested with the restriction endonuclease *Msp* I or *Rsa* I (Promega). The digestion was carried out in a total volume of 20 µl for 4 hours at 37°C. One µl of the digested amplicons was mixed with 14.4 µl of formamide and 0.6 µl of an internal DNA standard (GeneScan 2500 TAMRA, Applied Biosystems). The mixtures were denatured at 95°C for 2 minutes. Electrophoresis on a polyacrylamide gel was then performed using ABI373 XL DNA sequencer. The sizes of the 5'-terminal restriction fragments and the intensities of their fluorescence emission signals from the gel were calculated and analyzed by the GeneScan 3.1 and Genotype (Applied Biosystems). Each fragment of unique length was counted as one distinct species. Sorenson's index, $C_S=2j_N/(a_N+b_N)$, a

pairwise similarity coefficient, was used to determine similarities in species composition between each combination of two seasons. *J* is the number of common species in samples A (any one season) and B (any other season), a_N is the number of species in sample A, and b_N is the number of species in sample B.

2.10 Statistics.

S-Plus and the Viabcal program was used for approximating viability from culture data (Quang and Button 1998). Other statistical analyses were performed using the SAS software (version 8) or SigmaPlot (version 9) and employed a significance value of 0.05. Since sample numbers were less than 20 and a Gaussian distribution could not be determined, non-parametric statistical tests were used to examine the differences between two means (Mann-Whitney) or among several means (Kruskal Wallis).

3 Results

3.1 Seasonal components of Harding Lake oligobacterial activity.

Flow cytometry, HPLC and kinetic data are presented in Figs. 3 and 4. Photosynthesis rates from LaPerriere (2003) (Fig. 3, Panel B) are included to show potential substrate input over the year. The proportion of large bacterial cells which appear to be replicating (Fig. 3, Panels G through J) increases in early spring, coincident with photosynthesis, amino acid concentration and specific affinity of bacteria for amino acids. Table 2 compares the relative magnitude of changes in these seasonal properties over the annual cycle. Microbial biomass appears relatively constant compared to nutrient supply and activity fluctuates to control nutrient concentrations. Overall, the below-ice water column appeared well-mixed and populated by remineralizers (Fig. 4).

Flow cytometry and TRFLP patterns for surface lakewater show a near complete turnover of bacterial composition (Figs. 1 and 5) with about 10 species predominating at any one time. Cytograms display distinct DNA content and mass of each cell as a single event (Robertson and Button 1989). Although two cells of a single species may display differential DNA shedding (Moyer and Morita 1989; Vellai et al. 1999) or different genome number and biomass related to cell cycle, a single species population will appear in the same position, within the error of analysis, given similar growth conditions. Cytograms from the three different seasons analyzed show three distinct patterns. Sorensen's index was calculated for TRFLP peaks and indicates a dynamic bacterial composition. The similarity coefficient for the winter and spring samples was slightly higher than for the summer and spring samples (0.22 and 0.17 with *Msp* I; 0.20 and 0.17 with *Rsa* I) indicating greater similarity between winter and spring than between spring and summer. However, the similarity coefficient for the summer and winter samples was greatest (0.30 with *Msp* I and 0.26 with *Rsa* I). All coefficients were less than 0.4 and similar peaks were rarely identified, confirming a replacement of nearly all dominant species among seasons.

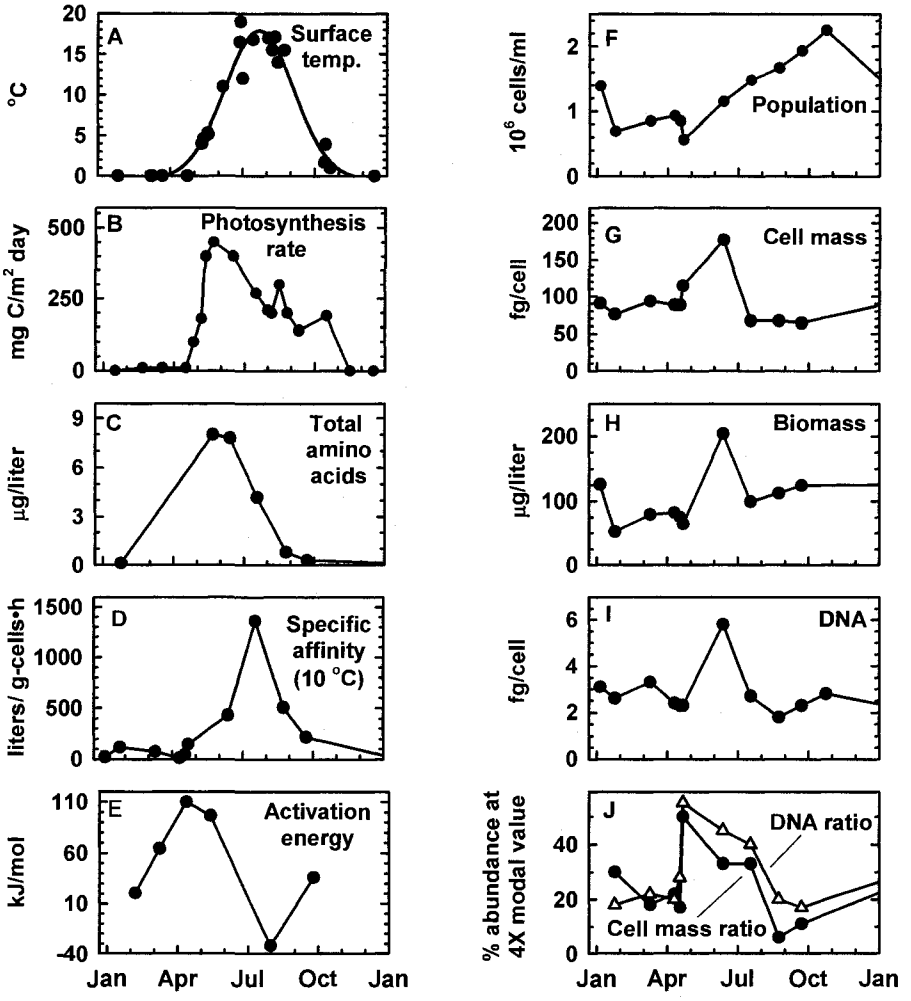

Figure 3. Seasonal properties of Harding Lake. Measurements are for surface samples except B, which is integrated over depth. A: temperature of surface water or water just below the ice; B: photosynthesis rate from LaPerriere, 2003; C: concentration of seven amino acids measured by HPLC; D: specific affinity calculated from respiration rates measured with 0.20 µg of ^{14}C-amino acid mixture L^{-1} and biomass in H, assuming a yield of 0.5 $g_{cell(dry)}$ $g_{amino\ acids\ used}^{-1}$; E: energy of activation for warming of surface water between 5 and 7 °C above in situ temperature; F: bacterial population; G: cell mass (wet weight) calculations from FCM forward scatter using a ratio of 0.18 dry weight to wet weight; H: biomass calculated from F and G; I: modal DNA content of bacterial cells calculated from DAPI-DNA fluorescence by flow cytometry; J: ratio of cell abundance at modal DNA content or cell mass to abundance at DNA content or cell mass four times that of the mode.

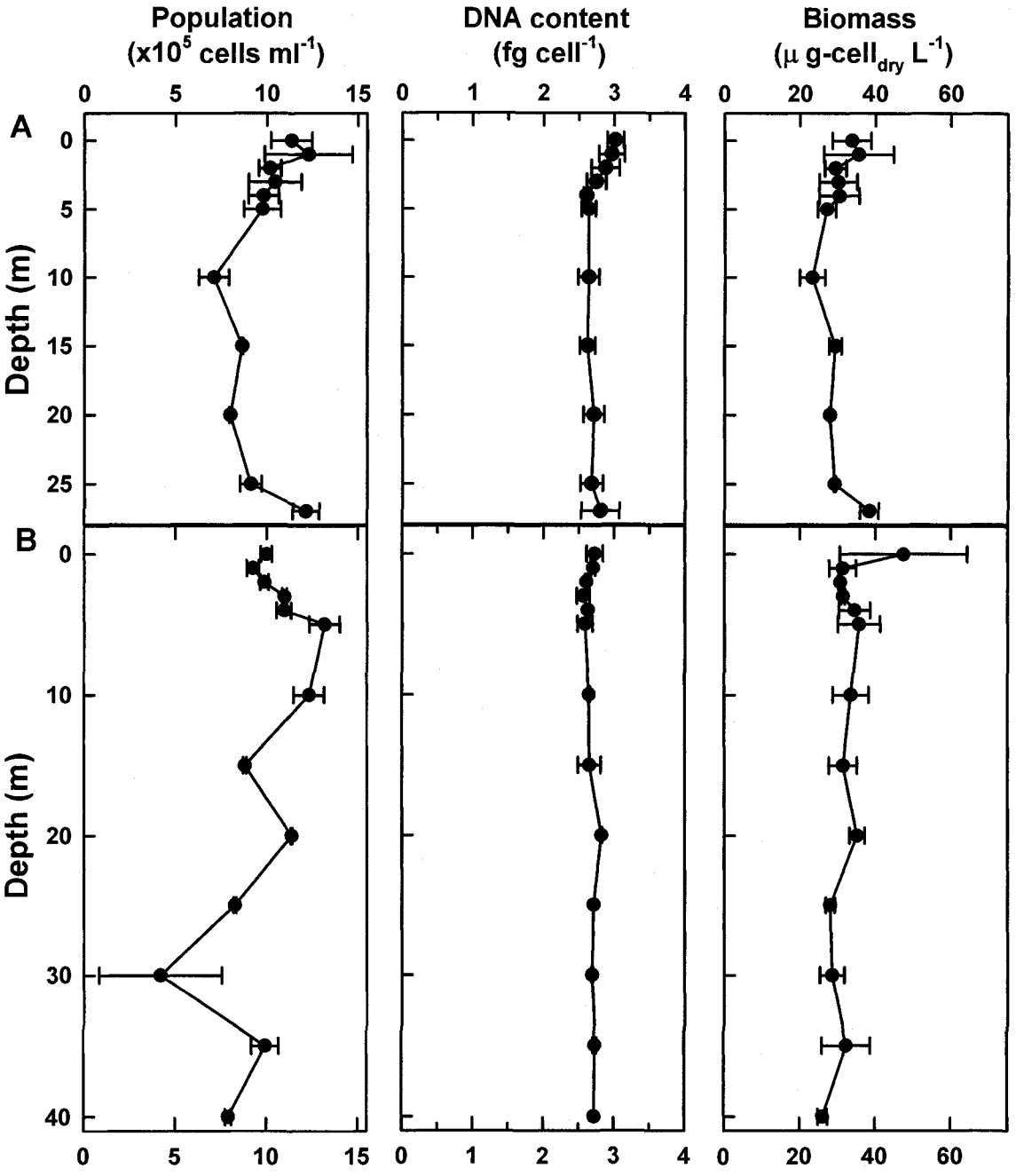

Figure 4. Harding Lake bacterioplankton properties for water samples from bottom of ice to lake bottom at two locations. A: 2/5/03; B: 4/17/03. n=2 and error bars indicate ranges except for A: DNA content data for which n=3 and error bars indicate standard deviations. Biomass is wet weight assuming a ratio of 0.18 dry weight to wet weight.

Table 2. Relative seasonal changes in parameters measured in Harding Lake. A relatively constant biomass serves as a flywheel for mineralization, matching its specific affinity for amino acids with changes in concentration.

Parameter	Seasonal change (factor)
Bacterial biomass	3
Bacterial specific affinity	≥300
Primary productivity	400
Total amino acids	7

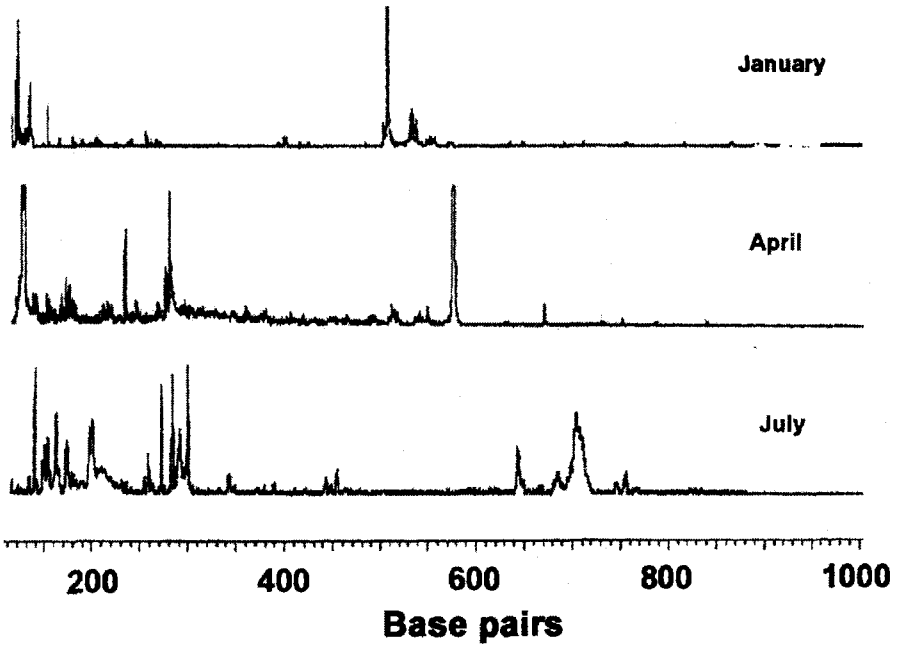

Figure 5. TRFLP electropherograms of Harding Lake surface water (Zhao 2005). Species compositions were different in Jan, Apr and Jul.

3.2 The bacterial flywheel.

Seasonal components are considered parts of a system which cycles minerals through a bacterial flywheel (Fig. 6). The energy and material transfers, viewed at one point in time, can be described by steady state formulae.

The rate of phytoplankton production, v_P, depends on both the input rate of minerals, v_M, and the availability of light energy, I. At steady state, predators such as zooplankton limit the population (Šimek et al. 2005; Williams 1995) so that the rate of phytoplankton production and the rate of their consumption are equal. However, because neither light energy nor grazing rates are constant, the standing stocks and primary productivities cycle around a yearly mean. The production rate of phytoplankton is

$$v_P = v_M \, f(I) \, Y_{MP} . \qquad (5)$$

Mineral consumption, v_M, depends on the growth rate μ_P and standing stock of phytoplankton P according to their mineral content Y_{MP}

$$v_M = \mu_P \, P \, Y_{MP} . \qquad (6)$$

Phytoplankton biomass, P, given ample light and other conditions appropriate for a bloom is given by the mineral (N, P, Fe) content M of the system

$$P = M \, Y_{PM} . \qquad (7)$$

The input of organic carbon v_S depends on herbivore damage to phytoplankton and associated leakage, rate constant k_L. If the removal rate by herbivores is equal to the growth rate of the phytoplankton μ_P, at steady state,

$$v_S = P \, k_L . \qquad (8)$$

Accumulation of substrates by bacteria of biomass X depends on the associated specific affinities a_S and the concentration S_i:

$$- v_S = \sum_{i=1}^{n} a^0_{S_i} \, S_i \, X . \qquad (9)$$

The concentration of organic substrates in aquatic systems is approximately constant and dependent on the rate of supply by phytoplankton and the ability of bacterioplankton to accumulate them.

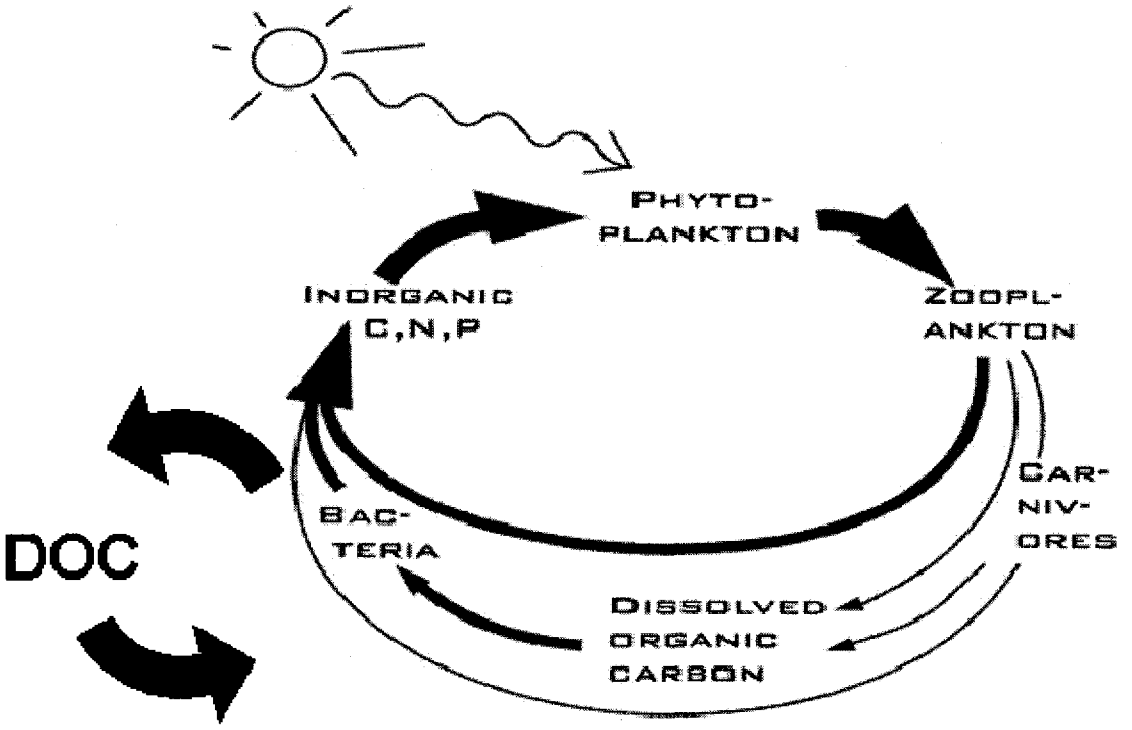

Figure 6. Mineral cycling in a sub-arctic lake. Bacteria act as a flywheel in an engine of mineral cycling. Radiant energy is stored in the summer as bacterial biomass. In winter, biomass is maintained by cycling with the pool of dissolved organic carbon (DOC) so that the springtime population is ready to respond to fresh DOC input from phytoplankton.

$$\frac{dS}{dt} = P\,k_L \quad - \quad \sum_{i=1}^{n} a^0_{S_i}\, S_i\, X \quad \approx \quad 0 \qquad (10)$$

Bacterial productivity is

$$\frac{dX}{dt} = \mu X. \qquad (11)$$

Specific growth rate (or partial specific growth rate on amino acids, μ_{aa}) is given by their consumption rate of organics from Equation 9 and the associated yield

$$\mu = \frac{v_S\, Y_X}{X} = \sum_{i=1}^{n} a^0_{S_i}\, S_i\, Y_i. \qquad (12)$$

Specific affinity of the grazers G/K_g is given by the maximal grazing rate by bacterivores G and the grazing constant K_g. Bacterial biomass X is strongly influenced by bacterivore grazing:

$$X = \frac{g\,K_g}{G - g} \qquad (13)$$

so that the population is insulated from the large variations that would otherwise be imposed by organic carbon input from phytoplankton blooms (Corno and Jürgens 2006; Šimek et al. 2005).

In Harding Lake, bacterioplankton response to small changes in nutrient concentration fluctuates with greater amplitude than phytoplankton production, substrate concentration or biomass (Table 2). Even if the highest specific affinity (1400 L g_{cell}^{-1} h^{-1}) is disregarded, values still vary by a factor of about 500, comparable to primary productivity. The relatively constant bacterial biomass acts as a flywheel in the cycling of minerals, persisting throughout winter and allowing efficient transition to rapid remineralization when richer conditions become available each spring.

3.3 Specific affinity values.

Specific affinity values presented in Fig. 3 D were derived from respiration rates of a ^{14}C-amino acid mixture at 10°C, and those used in Arrhenius equations were derived from incorporation rates of ^{3}H-amino acid mixture at in situ temperatures. To ensure temperature effects do not influence the interpretation of seasonal components and their

role in the bacterial flywheel, values from each method are compared in Fig. 7. Plots from each method follow a similar pattern and reflect reasonable year-to-year variations.

3.4 Sample treatment and uptake measurements.

Early incorporation rates (100 mL lakewater volume) with 150 mL bottles pre-soaked one day in lakewater resulted in perplexingly high coefficients of variation (average COV=40%, n=8), yet r^2 values were consistently large. Although the COVs are consistent with other published data (Ferguson and Sunda 1984; Yager and Deming 1999), incubation volume, pre-filtration and pre-soaking effects on rate means and precision were tested.

At least one pre-soaking treatment was significantly different ($F_{2(0.05),3}$=5.42; P=0.0250) and pre-soaking in various solutions decreased incorporation rates to between 80% and 85% of incubations with bottles not pre-soaked (Fig. 8). The COVs were similar among pre-soaked bottles and lower than initial measurements (COV=4.1 - 6.0%, n=3), presumably due to more careful manipulation of sample from collection to incubation.

Pre-filtering samples with GF/D glass fiber filters just prior to incubation decreased incorporation rates to 23% and 9.6% of the rates of samples not pre-filtered (Fig. 9). Generally, pre-filtration may remove much activity probably due to the removal of cells attached to particles greater than the pore size used (Button and Robertson 1989). However, since the two pre-filtered sets showed very different rates and the COVs were similar, the reduction could also have resulted from excess manipulation of the sample, perhaps by increasing the risk of contamination from exposure to extra glassware or air.

A ten-fold difference in incubation volumes had no significant effect on incorporation rates (P=0.6966). Table 3 shows the percent of total radioactivity incorporated per hour and the COV for each treatment set.

The incorporation rates were most reliable without pre-soaking of fired glassware and without pre-filtration of samples. Our COVs remained $\geq$ 30% for some experiments. The cause of error was not determined but was reduced with increased operator experience. All other incubations presented here were performed with unfiltered water in combusted glassware that was rinsed with sample just prior to use.

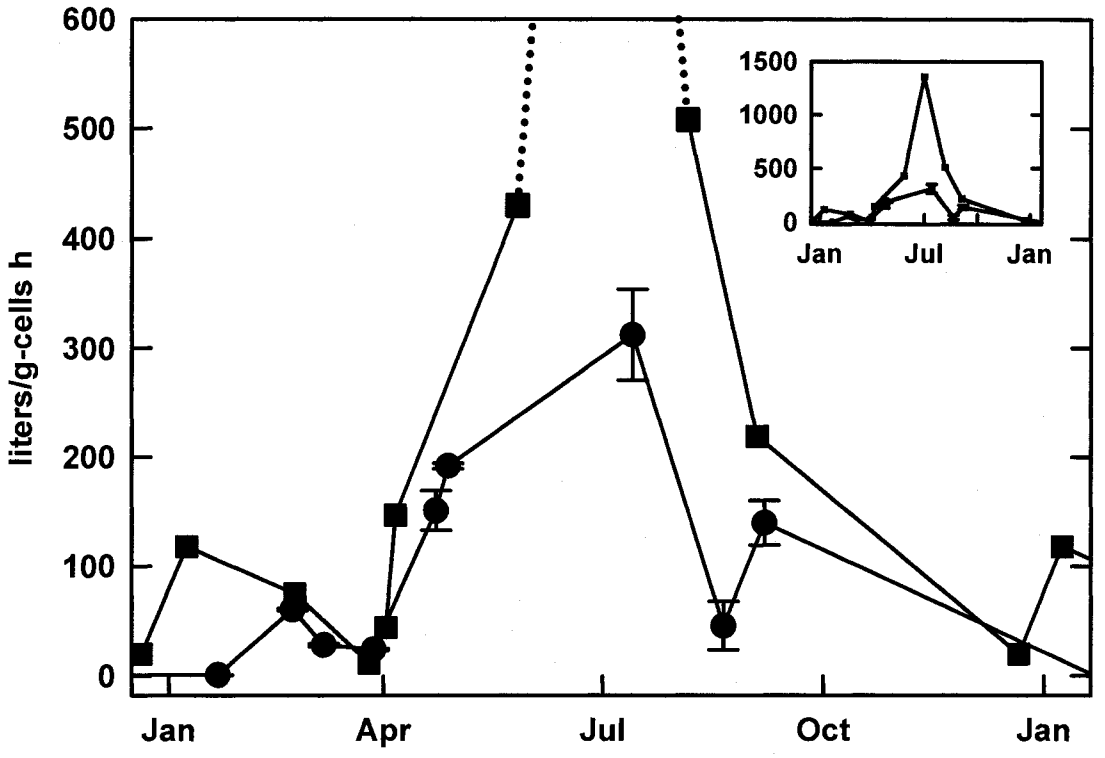

Figure 7. Seasonal specific affinity values obtained with different measurements. Squares: values derived from respiration rates of ^{14}C-amino acid mixture incubated at 10°C (1990-1991); July point is excluded in the large graph for finer ordinate definition. Circles: values derived from incorporation rates of ^{3}H-amino acid mixture incubated at in situ temperatures (2001-2003). Inset: all points included.

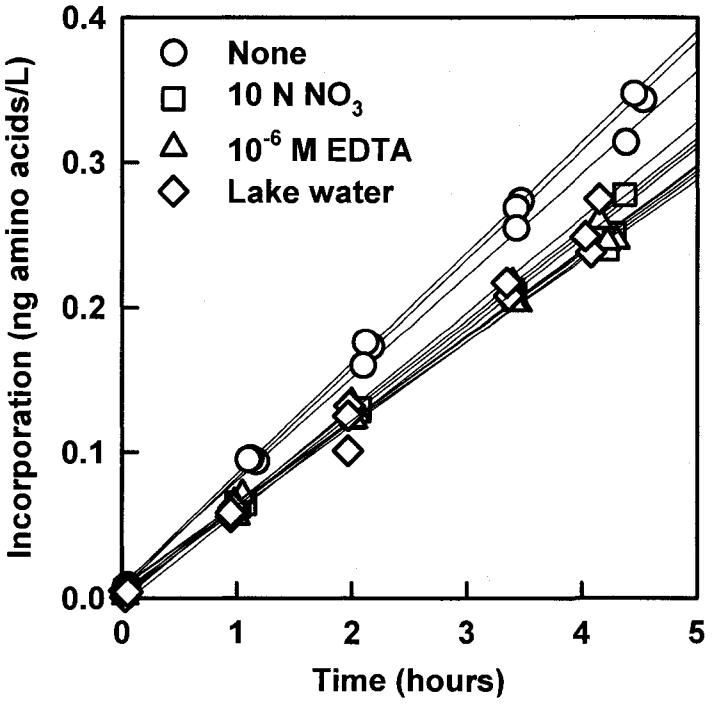

Figure 8. Incorporation of ^{3}H-amino acid mixture by surface water collected on 20 Oct 2000. Bottles were pre-treated by soaking with indicated solutions. Pre-soaking reduced incorporation rates by 15 to 20% (n=3) when compared to rates measured using bottles which were not pre-soaked.

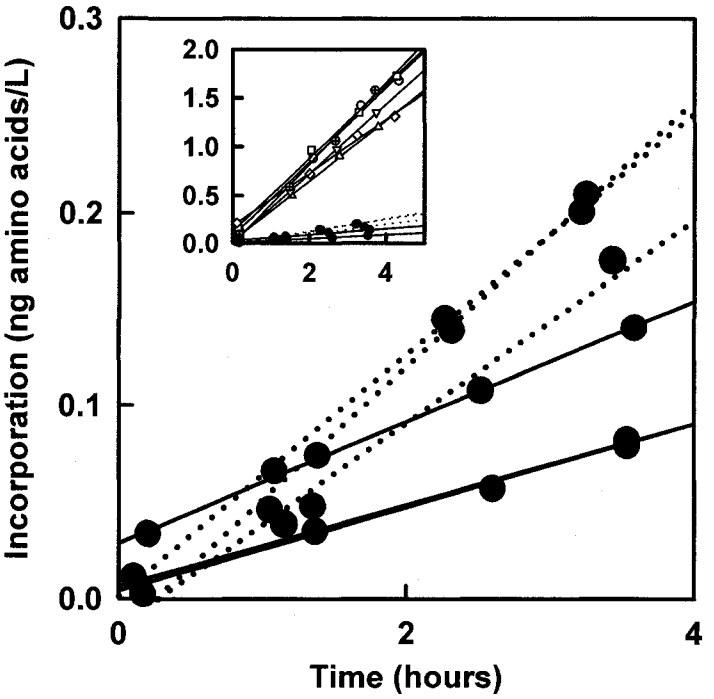

Figure 9. Incorporation of ^{3}H-amino acid mixture. Sampling date: 3/6/01. Ice thickness: 97 cm. Sample depth: 1.5 m below top of ice. Closed circles: GF/D filtrate, dotted lines are linear regressions for measurements from one filtration apparatus (n=3), solid lines from a second filtration apparatus (n=3). Open symbols: unfiltered samples (n=6).

Table 3. Bottle effects from incubation volume, sample filtration and bottle pre-treatment experiments. Lower coefficients of variation were obtained without pre-soaking of glassware and without pre-filtering the sample. Subsequent incubations were performed without pre-soaking or pre-filtration. COV is coefficient of variation.

Volume (n=6)	% of total radiolabel incorporated h^{-1} (COV)
1 L	2.0 (33%)
100 mL	1.9 (30%)

Filtration	% of total radiolabel incorporated h^{-1} (COV)
GF/D filtered, A (n=3)	0.032 (11%)
GF/D filtered, B (n=3)	0.077 (20%)
Unfiltered (n=6)	0.40 (18%)

Pre-treatment (n=3)	% of total radiolabel incorporated h^{-1} (COV)
None	0.31 (2.8%)
Lakewater soak	0.26 (4.7%)
Acid soak	0.26 (6.8%)
EDTA soak	0.27 (8.8%)

3.5 Yield.

Yields of cell material produced per substrate used ranged from 40% at 1°C to 70% at 6°C (Table 4; Fig. 10) and are similar to those found in marine systems for ^{3}H-labelled amino acids (Carlucci et al. 1984) and ^{3}H-glucose (Rich et al. 1996). Two experiments showed a 95.7% and 93.1% recovery of acidified $NaH^{14}CO_3$ (Table 5). Cell yields reported here are adjusted conservatively for 93% recovery of $^{14}CO_2$. The $^{14}CO_2$ evolution and ^{14}C incorporation rates were initially high, then decreased slightly after 4 hours, similar to the uptake rate incubations.

Rates were also similar in light and dark parallel incubations (α=0.05; P=0.973) (Fig. 11). Bacterioplankton previously thought to be heterotrophic have recently been found to contain light-harvesting rhodopsin genes (Béjà et al. 2001; Venter et al. 2004). If phototrophic bacterioplankton or phytoplankton were present in these samples, they 1) were not able to use $^{14}CO_2$ at the ambient light level used here, 2) were able to use $^{14}CO_2$ but it was replaced by some other mechanism, or 3) were not present in sufficient numbers to affect rates. Ambient light is too low for most phototrophy, so incubations in ambient light were likely unaffected.

To determine if isotope dilution impacted our yield values, cell yield was calculated for each time point in each experiment and plotted in Fig. 12. If CO_2 precursor concentrations were not rapidly equilibrated with $^{14}CO_2$, apparent cell yield would decrease over the incubation period. For some experiments yield appeared to be lower within an hour of incubation than after three hours. However, the potential for large error for early time points makes these measurements too uncertain to stand alone. For example, the 1 Sep 2005 experiment shows a yield of 0.15 after four minutes of incubation with radioisotope and 0.53 after 3 hours of incubation. Less than 10 DPMs mL^{-1} of sparged or filtered sample were counted at the earlier time for both respiration and incorporation; the 3-h time point measured over 200 DPMs mL^{-1}. If respiration rate increased in the first three hours (or cell yield decreased), specific activity was not great enough to resolve it. Furthermore, yield remained constant enough over the 3-h and 6-h measurements to lend confidence in the method used for final yield values. The use of a

Table 4. Yield values for Harding Lake surface water or water just under the ice.

Date	Temperature (°C)	Substrate (^{14}C-labeled)	Yield ($g_{cell\ carbon}\ g_{amino\ acid\ carbon}^{-1}$)
Feb 2005	1	Amino acids	0.40
Apr 2005	4	Amino acids	0.47
7 May 2005	6	Amino acids	0.70
1 Sep 2005	15	Amino acids	0.56
1 Sep 2005	15	Glucose	0.52
26 Sep 2005	11	Amino acids	0.66
26 Sep 2005	11	Glucose	0.70

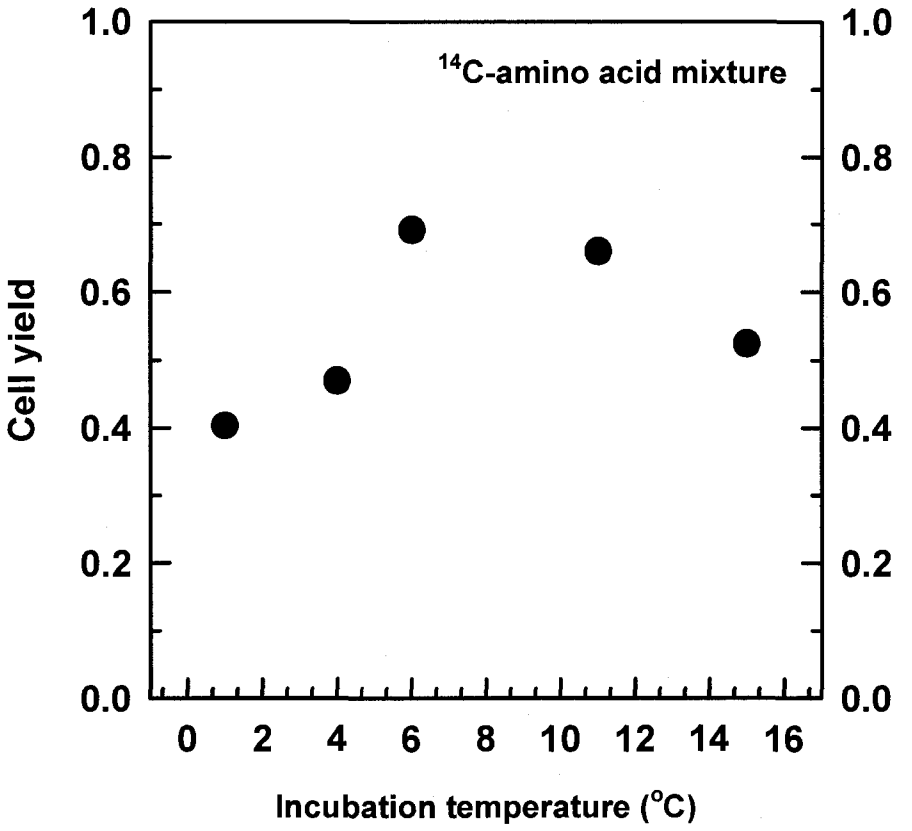

Figure 10. Cell yield for trace amounts of ^{14}C-amino acid mixture. Values are calculated as the proportion of radioactive cell material retained on 0.2 μm filters to cell material plus radioactive volatiles (presumably $^{14}CO_2$) evolved after acidification. Incubations were short (≤6 hours) and were performed at the in situ temperatures indicated. n=1 for 1 and 4°C experiments; n=2 for 6, 11 and 15°C experiments.

Table 5. Percent radioactivity recovered after acidification of $NaH^{14}CO_3$ by three vials of PEA in series. Dates indicate experiments. Yield data reported are corrected conservatively for 93% recovery of ^{14}C-volatiles.

Vial in series	1	2	3	Total recovery (%)
17 Aug 2005	92.70	2.87	0.11	95.7%
22 Aug 2005	90.00	2.70	0.52	93.1%

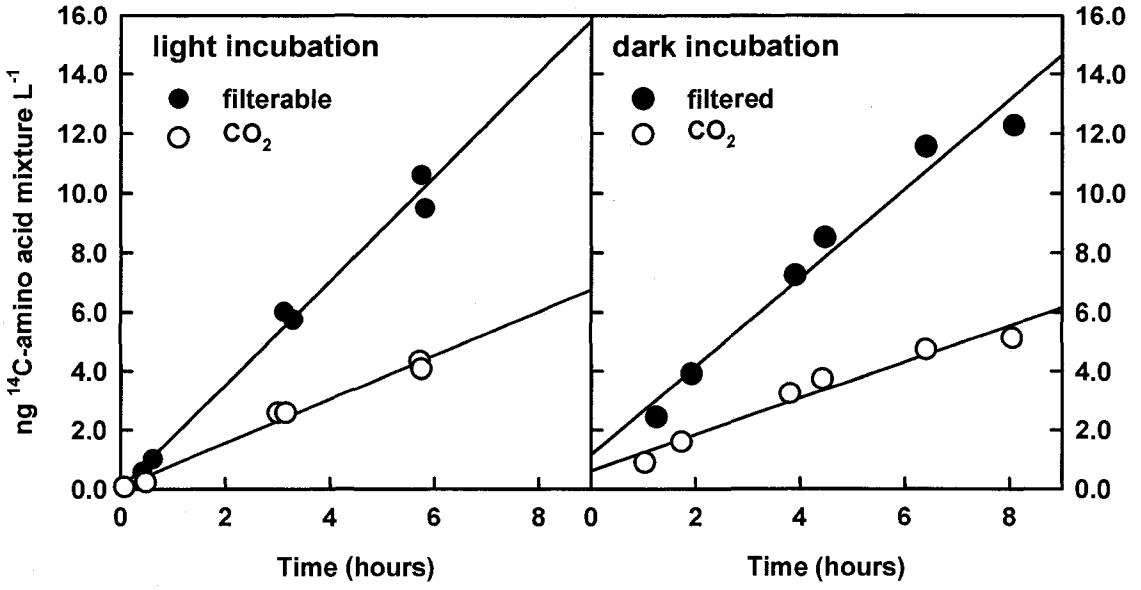

Figure 11. Parallel incubations in ambient light or dark (n=2). Measured DPMs from ^{14}C evolved or retained by the cells were converted to ng of labeled amino acids using the specific activity of the mixture and an average molar weight of 120 g (mol amino acids)$^{-1}$.

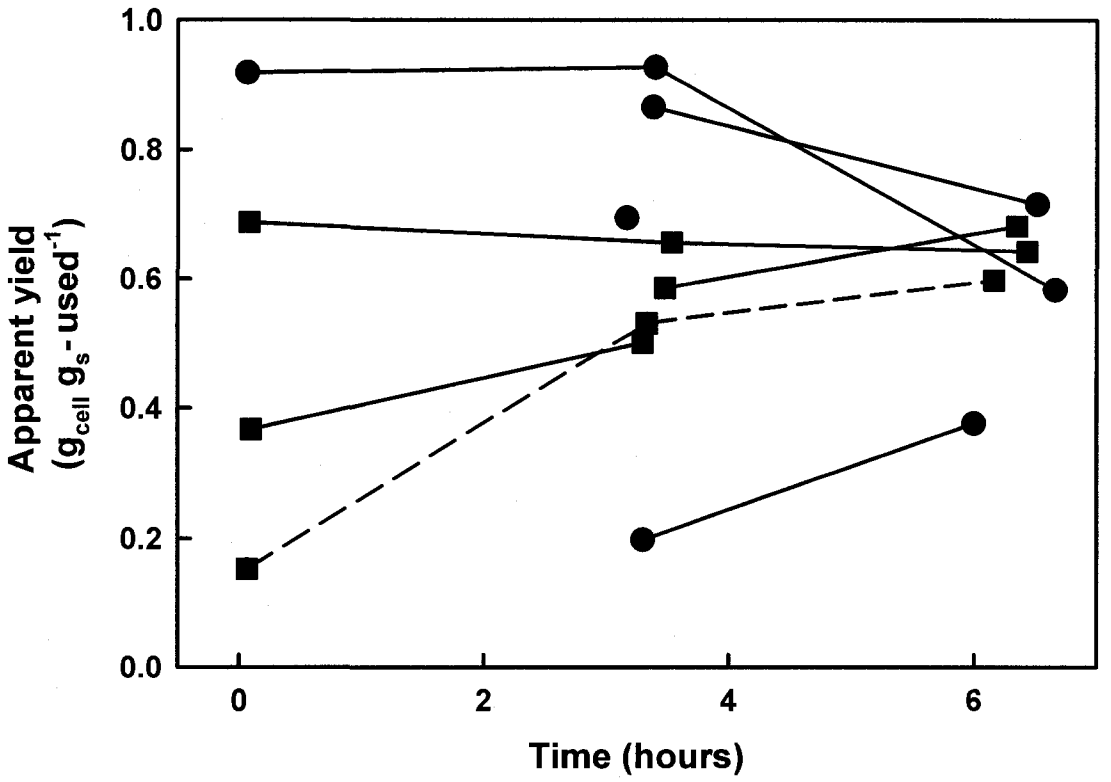

Figure 12. Apparent cell yield calculated for each time point during eight incubations. Circles: incubation with ^{14}C-glucose; squares: ^{14}C-amino acid mixture. Dashed line indicates the 1 Sep 2005 experiment noted in text.

CO_2 collection train for these yield measurements also minimized the potential for loss during acidification and recovery and provided a test point for the technique.

3.6 Culturability.

Apparent viabilities were calculated for all dilutions within treatment sets (Fig. 13; Table 1). Glass vessel cultures did not appear to benefit from substrate. Lyophil additions resulted in an estimated viability of about 5%, but sample number was low and error high. Diffusion chamber growth was greatly increased by amendment with glucose (73%) or amino acids (33%). However, this method may not reflect true culturability. The number of species arising in diffusion chambers exceeded the inoculum size (squares, Fig. 14). The dashed line in Fig. 14 indicates the expected outcome if all cells are viable and grow to a detectable concentration under the culture conditions employed; each cell in the inoculum is a different species; and the average inoculum size is the true inoculum size. The diffusion chamber experiments also produced a higher proportion of positive controls, 27.2% (s.d. = 20.7%) versus 4.4% (s.d. = 6.8%), for glass tube controls. This suggests membrane failure.

3.7 Temperature.

Bacterial populations showed a remarkable response to small (5-7°C) warming ($P<0.05$; Fig. 15). Activation energies were greatest (110 kJ mol^{-1}) in the springtime in 1°C surface water (Fig. 3 E) and smallest (-32 kJ mol^{-1}) in 15°C summer water. Most Arrhenius curves appeared at least biphasic with greater slope (and activation energy) at lower temperatures (Fig. 16).

3.8 Nutrition.

Specific growth rates (μ) were calculated with data from three different methods to estimate amino acid contribution to overall nutrition (Table 6). Values for organisms growing on amino acids alone, μ_{aa}, were calculated with Equation 12 and the substrate concentrations reported in Fig. 3 C. They range from 0.0563 day^{-1} in July to 3 x 10^{-5} day^{-1} in January with turnover times of 17.8 to 36,000 days, respectively. Specific growth rates, μ, for cultures arising in glass tubes from single cells were at least 0.35 day^{-1}. For a bacterioplankton population comprising 10 species (Fig. 5) to grow from 10^4 cells mL^{-1}

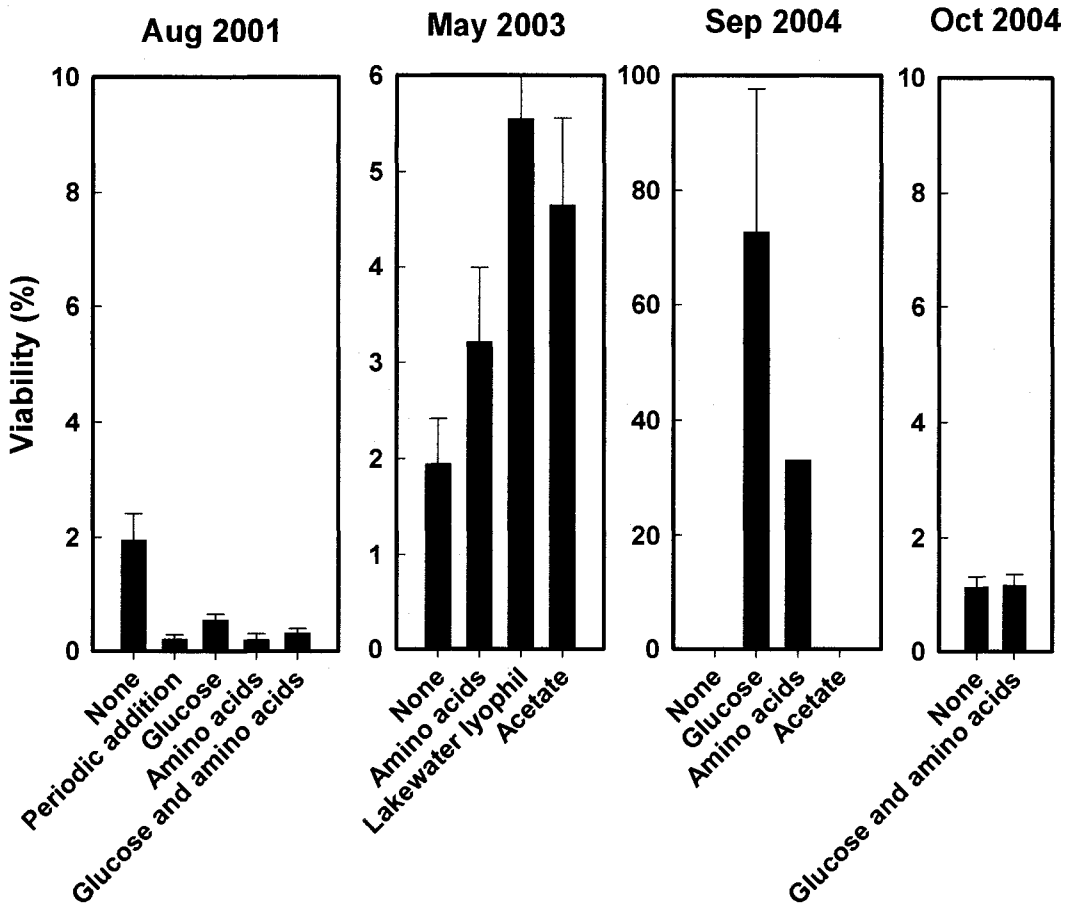

Figure 13. Apparent viability values for Harding Lake cultures. Aug, May and Oct experiments were extinction cultures in glass tubes. Sep experiment was performed in diffusion chambers. See Table 1 for amendment concentrations, inocula sizes and detailed viability calculations.

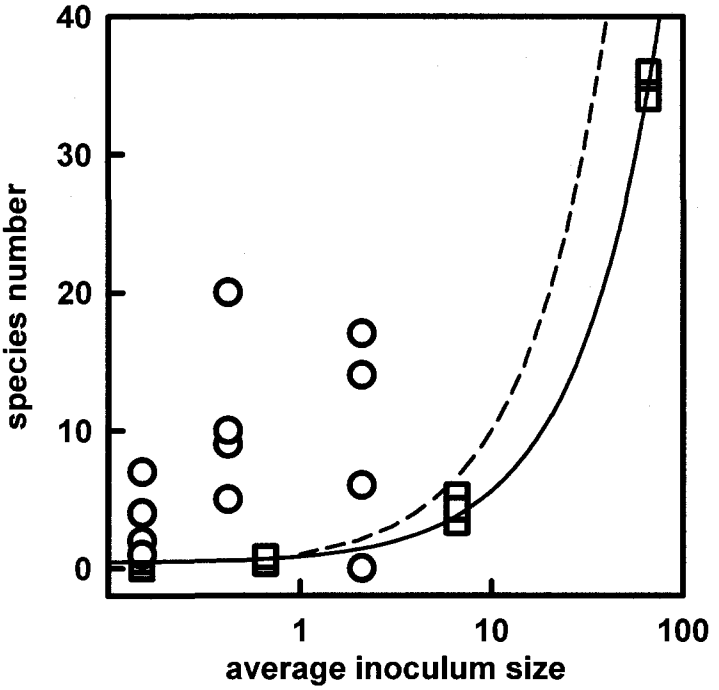

Figure 14. Numbers of species arising in cultures by average inoculum size. Species number was determined by TRFLP and is the average number of species arising from all positive cultures in the experimental treatment group. Glass tube cultures (squares); diffusion chamber cultures (circles). The solid line is the regression of the glass tube culture data. The dashed line is the expected outcome if only one of each species is included in each inoculum and all cells grow to a detectable concentration.

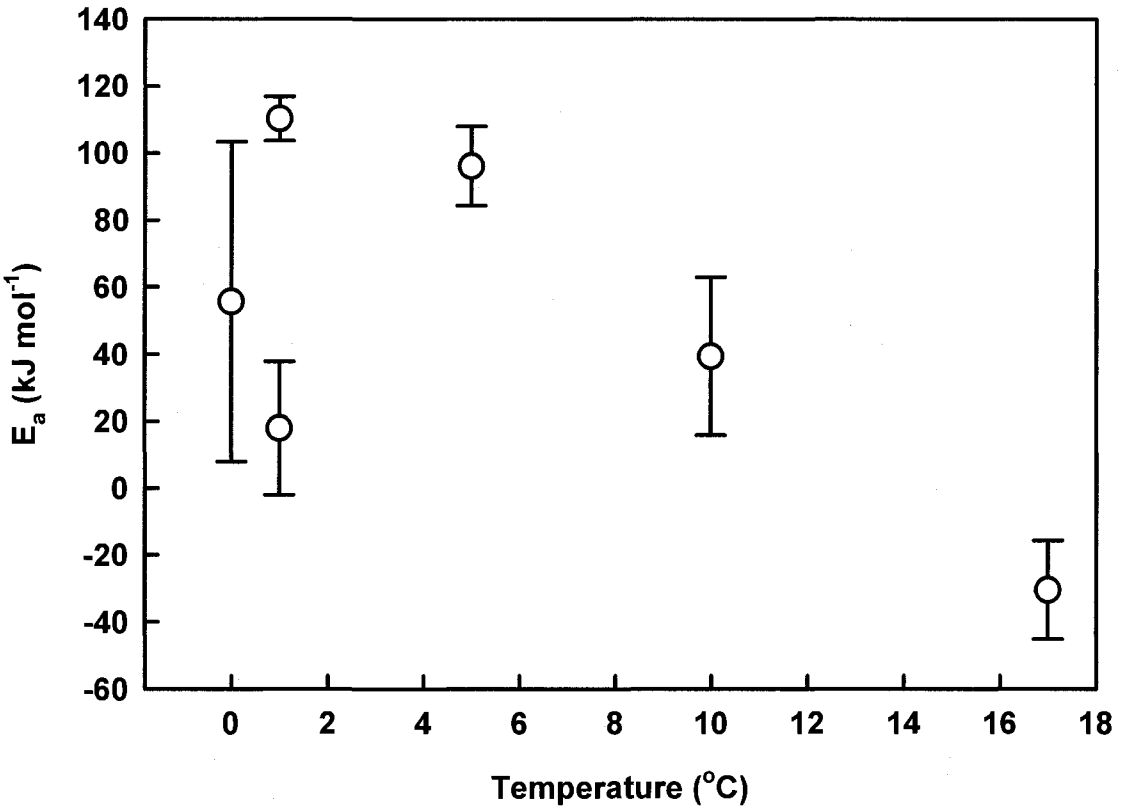

Figure 15. The ability of bacterioplankton to increase activity with warming as a function of in situ temperature. Incubation temperatures for each experiment were the in situ temperature and one temperature between 5 and 7 °C warmer. Apparent activation energies (E_a) were calculated from the regression lines of Arrhenius plots (K^{-1} vs. ln a_S) of the specific affinities for ^{3}H-amino acids mixture ($n \geq 2$ at each temperature). Error bars = std. err.

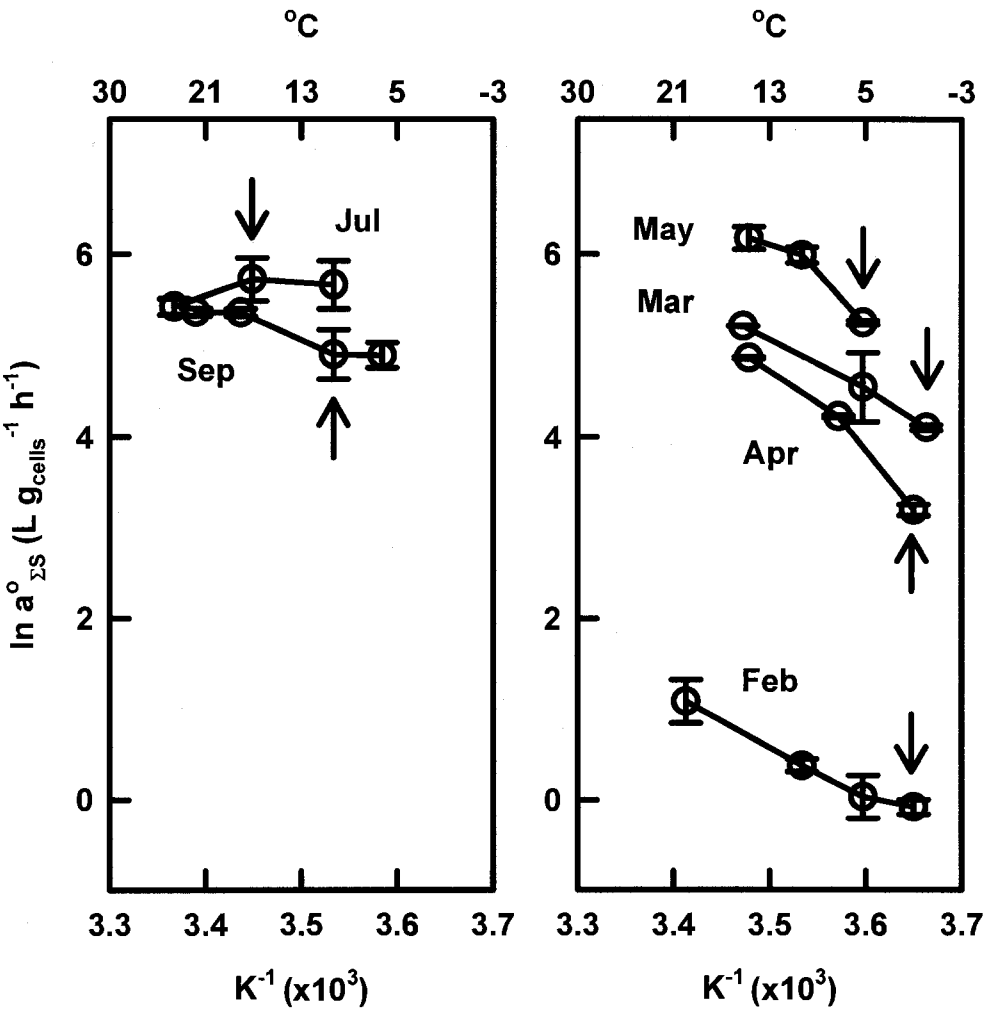

Figure 16. Arrhenius plots of specific affinities for ^{3}H-amino acid mixture. Incubations were 4-6 hours long. Samples incubated at in situ temperatures are indicated with arrows. Error bars indicate standard deviations (n>2) or ranges (n=2). Dates indicate single experiments.

Table 6. Seasonal properties of bacterioplankton dynamics.

	μ_{aa} or μ (day^{-1})	Population turnover time (days)	Doubling time (days)	Secondary productivity[d] ($\mu g_{cells}\ L^{-1}\ day^{-1}$)	Percentage of primary productivity[e]	Primary productivity ($\mu g_{cells}\ L^{-1}\ day^{-1}$)[f]
Radioisotope measurements[a]:						
January	0.000028	36,000	25,000	0.0032 (1.5 – 6.6)	-	0
April	0.0150	64.7	46.2	0.865 (0.75 – 3.3)	0.346 (0.3 – 1.4)	250
July	0.0563	17.8	12.3	5.04 (1.2 – 5.2)	0.747 (0.18 – 0.77)	675
September	0.0009	1110	770	0.101 (1.5 – 6.5)	0.0269 (0.4 – 1.7)	375
Extinction cultures[b]:	> 0.35	2.9	< 2.0			
Population turnover[c]:	0.013 – 0.027	77 - 17	53 - 12			

[a] Partial specific growth rates (μ_{aa}) were calculated from radioisotope measurements of samples incubated with a mixture of 15 ^{14}C-amino acids. Equation 12 was used assuming yield = 0.5; turnover time = μ^{-1}; and doubling time = (ln 2)/μ.

[b] Specific growth rates (μ) = (ln(final [cell]/original [cell])/time). Typical values for extinction cultures growing in unamended FAL were used: final [cell] = 3 x 10^5 cells mL^{-1}; original [cell] = 1 mL^{-1}.

[c] Calculations as for extinction cultures in footnote b, assuming final [cell] = 1.0×10^5 cells mL^{-1}, original [cell] = 10^2 or 10^4 cell mL^{-1} and time = 120 days. Note: 10^4 is the largest population of a single species that may be present without being detected by TRFLP, FCM or epifluorescence microscopy. The detection limit for TRFLP was determined with *E. coli* (Zhao, 2005).

[d] Secondary productivity = $dX/dt = \mu X$, in wet weight (as in equation 11). Biomass values are selected for indicated months from Figure 2. Partial specific growth rates, μ_{aa}, from radioisotope measurements (first column) were used. Values in parentheses were calculated with specific growth rates from TRFLP data (see footnote c).

[e] Ratio of secondary to primary productivities in μg of cells (wet weight) L^{-1} day^{-1} and expressed as a percentage.

[f] Photosynthesis rate units were converted from area to volume using the fact that most activity occurred in the top 4 m of water. Assumes carbon is half of dry weight of phytoplankton and 0.2 g dry weight per 1.0 g wet weight phytoplankton cell.

(the detection limit of TRFLP) to 10^5 cells mL^{-1} within six months, a minimum growth rate of 0.013 day^{-1} (turnover time of 77 days) on amino acids is required (see footnote c, Table 6). This value of μ is close to the partial growth rate values, μ_{aa}, calculated for April and July populations but not for January or September populations. Secondary productivities calculated with Equation 11 and μ_{aa}s for all seasons were less than 1% of primary productivity, much lower than those reported in the current literature.

Specific affinities for amino acids did not respond to exogenous glucose at any of the applied concentrations nor with warming ($P>0.05$; Fig. 17). However, yield data show that organisms were able to use glucose in September. Uptake rates for the 26 Sep 2005 yield experiment were calculated as a fraction of added label. Values were 0.0589 h^{-1} for glucose and 0.0581 h^{-1} for amino acid mixture (n=2 each). On 1 Sep 2005, glucose specific affinity was 55 L g_{cell}^{-1} h^{-1} (n=1) compared to 45 L g_{cell}^{-1} h^{-1} (n=2) for amino acid mixture. In situ glucose concentrations were not measured in this study but have been below detection limits for other researchers at this study site (LaPerriere 2003).

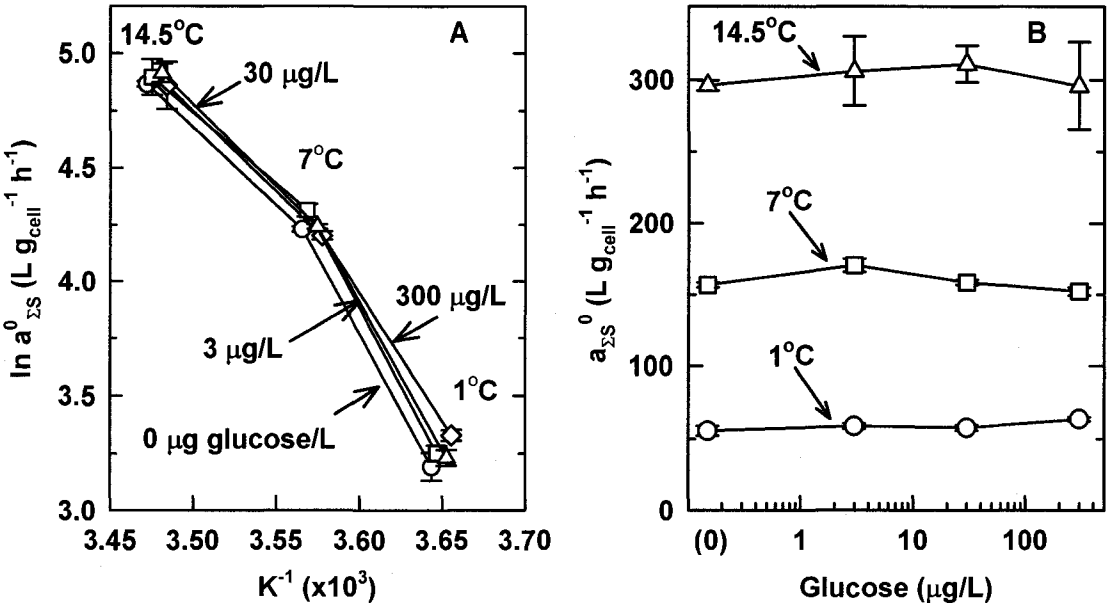

Figure 17. A: Arrhenius curves and B: specific affinities for ^{3}H-amino acids from an April warming experiment. Parallel incubations with cold glucose are included at each temperature and concentrations are noted with arrows. In situ temperature=1 °C; n=2; error bars represent ranges. Similar experiments were performed in March, May, July and September with similar results.

4 Discussion

4.1 System fragility.

'Bottle effect' is the term used for various effects of containment on aquatic samples. The nature of bottle effect is still a matter of conjecture among researchers, but variable data are known to result from the use of only slightly different techniques and equipment. For example, incubation volume can affect bacterivore grazing rate (Marrasé et al. 1992), a 'clean' sampling method and bottle type can affect amino acids turnover (Ferguson and Sunda 1984), and incubation time changes bacterioplankton species composition (Ferguson et al. 1984; Šimek et al. 2005), decreases bacterivore grazing rates (Marrasé et al. 1992) and increases amino acid turnover (Ferguson et al. 1984; Ferguson and Sunda 1984). Until sources of variability are identified, a conservative approach seems prudent and is achieved by minimal and careful manipulation of samples from collection through incubation, the use of subsaturating concentrations of labeled substrate and incubation times less than 6 hours.

4.2 Temperature response.

Measures of activity have been compared to incubation temperature for whole organisms (Bakermans and Nealson 2004; Mohr and Krawiec 1980). Arrhenius profiles like those in Fig. 16 illustrate temperature relationships more thoroughly than optimal growth temperatures. Here, we use one part of the Arrhenius curve, from the in situ incubation temperature to between 5 and 7 °C warmer, to quantify the response by mixed bacterial communities to small warming. Although apparent activation energy (E_a) is usually calculated only for the part of the Arrhenius curve below the optimal temperature, July's negative E_a is included as a seasonal value for comparison (Fig. 15). It should be remembered that E_a for whole organisms reflects more than the thermal properties of individual enzymes (Thomas and Cavicchioli 2000).

Breaks in Arrhenius curves, such as those in Figs. 16 and 17, have been found in published warming experiments. They have been interpreted as temperature minima, maxima and optima for growth (Mohr and Krawiec 1980). Also, they have been related to membrane properties such as fluidity (Eze and McElhaney 1987) and phase transitions

(Linden et al. 1973), and to physiological processes such as RNA production and cell yield (Bakermans and Nealson 2004). Arrhenius curves similar in profile to those presented here have been found with fatty acid auxotrophs (Eze and McElhaney 1987), environmental isolates (Bakermans and Nealson 2004; Pomeroy and Wiebe 2001) and membrane vesicles (Shechter et al. 1974).

The highest activation energies in Harding Lake (110 kJ mol^{-1}; Fig. 3 E; Fig. 15) were much higher than those of simple enzyme systems but were similar to those demonstrated in marine systems (Yager and Deming 1999). Computations showed that any step in a metabolic pathway that recycles energy from a component step to increase the equilibrium constant for that step can, if that step is accelerated by warming, increase the apparent activation energy for the whole pathway. In the present case amino acids are transported across the cytoplasmic membrane up a large concentration gradient either at the expense of ATP or of a membrane potential. In either mechanism the empowering currencies include protons. These are ejected by respiratory enzymes from the cytoplasm into the periplasm and used to drive active transport. Both protons and amino acid molecules are co-required for the transport step. At low temperature and low concentrations of amino acids this "bi-uni" reaction may either lack the protons for the molecular pump to operate, allow it to operate only slowly, or perhaps transport relies on diffusion alone. Warming may cause the inherent activity of the pump to increase. However, the additional protons from dismutation of the transported substrate can add to the likelihood that a substrate molecule will be transported due to necessary cooperation between the proton and the amino acid molecules. This positive cooperativity often results in a change in the kinetics from hyperbolic to sigmoidal as given by the Hill equation. It specifies that rate is slow at small concentrations but increases sharply at intermediate concentrations until saturation prevails as in the classic case of oxygen binding by hemoglobin.

Individual enzyme properties were not measured in this study but are known to influence activities as well. Cold adapted enzymes have higher activity at low (0-30°C) temperatures than their mesophilic or thermophilic counterparts, perhaps due to greater

flexibility. These benefits are counterbalanced by a compromise in thermostability. Multiple structural features are identified as contributing to a less stable, more flexible structure including loss of salt bridges, amino acid composition (Johns and Somero 2004) and the presence of extended loop structures (see review by Feller et al. 1996).

Activation energies in cold February water (0.5°C) and water ≥10°C were comparatively small, <40 kJ mol^{-1}. February organisms operate at the lowest temperatures experienced by Harding Lake bacterioplankton. Their smaller response to warming suggests a strong adaptation to very low temperatures. Substrate concentrations are also low in February so adaptation to cold temperatures with low specific affinities may conserve a small nutrient pool during winter. Small activation energies in warmer summer water may result from protein denaturation and leaky membranes of cold adapted cells at supraoptimal temperatures, similar to cold adapted laboratory isolates (Feller et al. 1996). These data show that Harding Lake bacterial populations are comprised of different species consortia over the annual cycle, which are well adapted to different but narrow and low temperature ranges when compared to those of typical isolates.

4.3 Heterotrophic activity.

Amino acids are known to comprise a significant portion of bacterioplankton nutrition (Andrews and Williams 1971; Rosenstock and Simon 1993; Volk et al. 1997). Specific affinity for amino acids is used here to track changes in metabolic activity with treatment (incubation technique, warming, exogenous glucose) and over seasons. Several points must be considered when interpreting calculations from amino acid uptake data collected from natural communities: 1) some unknown portion of cells may not take up amino acids, 2) some portion of cells may be temporarily inactive or dead, 3) the proportion to which individual cells take up amino acids compared to other substrate types may vary, and 4) cell yield varies by substrate type and physiological state.

The first point may be determined to some degree by autoradiography. This was not performed in this study but the literature points to a widespread use of amino acids by free pelagic bacteria (Karner and Fuhrman 1997; Ouverney and Fuhrman 1999; Simon

1985). Karner and Fuhrman (1997) reported that close to half the number of DAPI staining cells in coastal surface water were detectable by autoradiography with labelled amino acids. Ouverney and Fuhrman (1999) reported that 70 to over 90% of DAPI staining cells in coastal surface water took up amino acids. Tabor and Neihof (1982) found that at least half of the acridine orange staining cells from Chesapeake Bay water utilized amino acids. Simon (1985) found that between 5.0 and 88.5% of the number of acridine orange stained cells in Lake Constance utilized amino acids to a level detectable by autoradiography. Interpretation of autoradiography results is complicated by the high concentrations of radioisotope needed to detect uptake by single cells. High concentrations may induce activity that does not occur naturally. Also, uptake is detectable only for cells which contain enough radioisotope to produce a silver grain so that cells with lower uptake rates, which may be numerically significant in low nutrient environments, are not detected. Therefore, percentage values may be taken as minima under conditions which may be saturating for much of the population.

The second point (some cells are inactive or dead) is addressed by the extinction culture work presented in this study. Estimated viability was indeed low for each experiment (0-10% for culture tube experiments). Generally, if culturing conditions are appropriate to reflect in situ growth then over 90% of inoculated cells should be considered inactive or dead (Fig. 13). This is unlikely because very few genomes detected by partial sequencing of 16S rRNA genes in marine and fresh water are represented in culture collections (Bruns et al. 2002; Connon and Giovannoni 2002; Giovannoni and Stingl 2005; Suzuki et al. 1997). Studies using techniques similar to those used here still result in the cultivation of a small percentage of species present. This probably reflects an incomplete understanding of appropriate culture conditions for these organisms.

One possible reason for the discrepancy between culturability and the number of species arising in culture is that the culture media are somehow inappropriate or insufficient to allow growth of the more fastidious organisms. Media which employ natural water are often filtered through 0.2 μm pore size membrane filters and then heat

sterilized. This is necessary to ensure media are not enriched by broken cell material and that only inoculated organisms have the opportunity to grow. In this study, we first used lyophil addition to filtered autoclaved lakewater in glass tube cultures for the purpose of enriching cultures with substrates which they naturally encounter. The results were not conclusive due to the small sample size. As an alternative to and an improvement over this technique, we used diffusion chambers to provide cultures with access to potential growth requirements via a small pore membrane. Cultures were also illuminated to provide energy to any light-driven processes which may be present in the whole lakewater.

Although the occurrence of positive controls greatly complicates the interpretation of the results in the diffusion chamber experiment, the technique addresses important issues and deserves further attention. If in situ viability values are of interest, culture conditions should resemble in situ conditions as closely as possible. Diffusion chambers simulate in situ processes more closely than any other technique currently in use. Previous work in seawater has shown an improvement in apparent viability in seawater (2 to 60%) and resulted in the identification of two new bacterioplankton, *Sphingomonas alaskensis* and *Cycloclasticus oligotrophus* (Button et al. 1993; Button et al. 1998; Schut et al. 1993). More detailed genomic work would prove useful in determining interactions between species as well as the relative culturability of dominant species.

The degree to which individual organisms in natural populations utilize amino acids compared to other substrates cannot be directly measured. However, the nutritional composition of natural communities as a whole can be deduced from specific growth rates. For example, the partial specific growth rates, μ_{aa}, for bacteria consuming amino acids alone and the rate required for species turnover, μ, are very different in winter (Table 6). Some possibilities exist to explain this disparity. Substrate concentrations may be low enough in winter to eliminate membrane potential sufficient for active transport. This would necessitate diffusion processes for sustained slow growth, processes that would not be detected by amino acid uptake experiments. Also, since data were obtained with the two different methods during different years, the extent of annual variation in

specific affinities was examined for the 1990-1991 and the 2001-2003 time periods (Fig. 7). The patterns for the two intervals are similar with some differences occurring during summer and winter. However, the largest discrepancies between growth rates for the time points selected in Table 6 are in April and September, times when specific affinities agree quite well across years. Therefore, annual variation in specific affinities is not likely the primary source of disparity between growth rates obtained by the two methods. Bacteria must also utilize substrates other than amino acids to meet the requirements of the observed population turnover (Law and Button 1977), and utilization of 'other' substrates may vary seasonally.

Point four is addressed by the measured values of cell yield for amino acids and for glucose. Early laboratory work with isolates found remarkably constant cell yield values near 0.6 (Payne 1970). Incubation of environmental samples with radiotracers has been criticized as a yield determining method because intracellular isotope dilution, specifically CO_2 precursor concentrations, is not typically addressed (Jahnke and Craven 1995; King and Berman 1984). Here, cell yield for Harding Lake samples is calculated from radioactivity distribution, similar to early field measurements (Crawford et al. 1974) and resulted in values similar to those reported in Payne, 1970. Within the precision of the method used, isotope dilution was not shown to impact final yield values in Harding Lake.

Cell yield for isolates and/or aquatic samples has been shown to vary by substrate type (Crawford et al. 1974), growth rate (del Giorgio et al. 1997; Pirt 1965), temperature (Bakermans and Nealson 2004; Knoblauch and Jørgensen 1999) and primary productivity (del Giorgio et al. 1997). For whole Harding Lake surface water, correlation with substrate type is not possible to assess since amino acids were used as a mixture. However, yield was highest in May and September, times when primary productivity and specific growth rate were relatively high, consistent with current literature. Yield was plotted against temperature to assess their relationship (Fig. 10). The relationship is nonlinear with the highest yield in 6°C water. This is similar to results with cold-adapted isolates. For example, sulfate-reducing bacteria isolated from Arctic sediments displayed

a variety of temperature-yield curves (Knoblauch and Jørgensen 1999). Some strains showed a nearly constant yield between the temperature at which they were isolated (-1.8°C) and their optimum temperature for growth. Some strains had highest yield around 0°C. *Psychrobacter cryopegella* also had optimal yields at low temperatures (Bakermans and Nealson 2004). Yield reflects an organism's metabolic needs and adaptations to current conditions. In Harding Lake, growth rates are unknown but specific affinities for amino acids are between 100 and 1000 times lower in winter than in summer (Fig. 7). Growth rates are the product of specific affinity for all substrates, substrate concentrations and yields (Equation 12). Winter amino acid concentrations are two times lower, which translates to a partial growth rate of about 1000 times lower in winter than in summer. While this large difference is probably ameliorated by a shift in substrate type, winter values are further compromised by both cooling (Fig. 15) and leakage. Rates of endogenous metabolism by conventional isolates are commonly near 0.06 $g_{acetate}\ g_{cells\text{-}dry}^{-1}\ h^{-1}$ (Tros et al. 1996). At this rate, endogenous losses would consume the bacteria of Harding Lake in 17 hours whereas wintertime populations probably sustain for weeks. Evidence for this can be found in part by the rather large wintertime yields found in combination with the very low specific affinities.

4.4 Nutrition.

Although the specific growth rates calculated from TRFLP data apply only to the bacterial population as a whole and to a few points in the annual cycle, the bacterial flywheel formulae and specific affinities provided by this study do provide a useful framework for examining nutritional composition in a complex system. The minimum growth rate required for the population turnover observed by TRFLP is at least 0.013 day^{-1}. In July, when the specific growth rate is 0.0563 day^{-1} growth may be entirely supported by amino acids. In contrast, January's partial specific growth rate is 0.000028 day^{-1} and amino acids comprise 0.22% of the observed minimum rate (100% x 0.000028/0.013 = 0.22%). If each substrate used has similar activity, then nearly 5900 more substrates are required to complete the observed minimum growth (100 x 13/0.22 = 5900).

Such considerations can direct further investigations concerning the identity of 'other' substrates used by bacterioplankton. For example, substrate fluxes were measured for both amino acids and glucose in September, a time when partial growth rate for amino acids is only 0.0009 day^{-1} and total amino acids concentration is around 1 $\mu g\ L^{-1}$. Amino acids account for 6.9% of the observed minimum growth rate, so 175 other substrates may be used (100% x 0.0009/0.013 = 6.9%; 100 x 13/6.9 = 188). However, if glucose is the only other available substrate, a concentration of 13 μg glucose L^{-1} and an absence of substrate saturation would be required. Glucose concentrations were not measured here but similar values have been measured in marine surface water (Andrews and Williams 1971; Rich et al. 1996). Winter values are likely lower so that January organisms rely on more complex substrates and intracellular reserves for maintenance and growth.

Simple components of bacterioplankton nutrition have been described elsewhere. Carbohydrates (Myklestad and Haug 1972) and amino acids (Lampert 1978; Larsson and Hagström 1979; Mague et al. 1980) are potential products of phytoplankton leakage and cell damage and are also sources for cell growth. Polysaccharides appear to comprise an active component to carbon cycling (Borch and Kirchman 1997; Kirchman et al. 2001; Pakulski and Benner 1994). Glucose is the most active component of the monosaccharide fraction. For example, in the equatorial Pacific glucose was measured from about 1 – 20 $\mu g\ L^{-1}$ with turnover times of up to about 0.6 day^{-1} (Rich et al. 1996). In the English Channel, up to 5.7 μg glucose L^{-1} were measured in summer with oxidation rates of up to 50% (Andrews and Williams 1971). Proteins may also be important substrates for bacteria with an uptake mechanism apart from that for free amino acids (Hollibaugh and Azam 1983).

A large portion of the bacterial growth observed in Harding Lake, particularly in January, must be supported by many different simple substrates, such as monosaccharides and polysaccharides, by some combination of more complex substrates, which are more difficult to quantify, or by some combination of the two. Amino acids were the only simple substrates measured in this study and the identification and fluxes of complex molecules await improvements in detection methods.

4.5 The bacterial flywheel and the microbial loop.

The current microbial loop paradigm was originally proposed as a challenge to the assumption that bacteria in the water column were primarily remineralizers of dissolved organic matter (Azam et al. 1983). Detailed examination of processes involved in the microbial loop are ongoing (Barber 2007) but most variations of the microbial loop still provide limited descriptions of contributions by heterotrophic bacteria to the conversion of organic matter to biomass at higher trophic levels (Landry et al. 1997).

The bacterial flywheel describes bacteria as both remineralizers and facilitators of carbon transfer to higher trophic levels but emphasis is on adaptations to prevailing seasonal conditions, which allow conservation of system energy as bacterial biomass. The use of specific affinity theory in the bacterial flywheel provides a link to cytoarchitectural properties of organisms (Button et al. 2004). This approach is unique in taking a bacteriocentric view of mineral cycling while accounting for grazing pressure and photosynthetic food supply. The model is particularly appropriate in high latitude environments where solar energy input is interrupted for long periods (winter). In more temperate environments where seasonal changes are of smaller magnitude, low nutrient supply to phytoplankton rather than solar input is more likely responsible for any interruption to mineral cycling. If interruption to cycling is long enough, the bacterial flywheel may act to maintain a biomass, which is known to remain above 10^5 cells mL^{-1} (Cho and Azam 1990), although temperature adaptations are likely different from their high latitude counterparts.

The bacterial flywheel has limitations, which must be kept in mind when interpreting experimental data. First, technical limitations make the response of individual organisms (such as changes in permease or cytoplasmic enzyme content and temperature adaptations) to environmental variables indistinguishable from changes in species consortia. Second, while the bacterial flywheel can be isolated in time as a series of steady states, it operates as part of an open system and many potential influences are unknown. For example, few specific growth requirements are known so that attempts to

isolate individual species from in situ mineral cycling and culture them in the laboratory (Fig. 13) are still largely unsuccessful.

When adaptations to seasonal changes are subscribed to both species succession and shifts in organism physiology, at least three different water types can be described: 1) In winter, temperature and substrate supply are low. Organism adaptations are high activation energies, low specific affinities for substrates and low rate of endogenous metabolism. These properties help winter organisms conserve energy reserves for springtime's increases in temperature and substrate concentrations and also for a slow replacement of species. 2) In spring, temperature and substrate supply transition upward. Specific affinity increases rapidly as well to optimize energy collection. Organisms present in spring possess energized enzymes which contribute to a very high activation energy and which optimize warmer temperatures. 3) Summer organisms have high specific affinities, controlling substrate concentrations supplied by high phytoplankton flux. Activation energies are low since a positive temperature response at the highest temperatures experienced by the organisms would be unnecessary.

4.6 Evidence for the bacterial flywheel in Harding Lake

Data presented in this study support the bacterial flywheel model. During winter, heterotrophic bacterial populations decreased only about 4-fold, while primary productivity was eliminated. Specific affinity of the bacteria for amino acids, as a measure of the activity of individual cells, decreased 100-fold or more. While there could have been a shift in nutrient preference, the large decrease for this ubiquitous substrate suggests a significant decrease in ability to accumulate nutrients. The bacteria also contain usual amounts of DNA and light-refractive solids (Button and Robertson 2001) with little reduction over winter. Both are normally related to the rate of growth with size corresponding to the enzyme content for a particular rate of growth and DNA content revealing additional genome copies per organism. These data and the continuous temporal shift in the distribution of major species occurring in winter low-affinity populations in the absence of any major population decrease suggest ongoing growth, a key component in the flywheel model.

There also appears to be a general shift in nutrient acquisition strategy during winter. Decreased cell yields are generally taken as a measure of the rate of endogenous metabolism, but while specific affinities decreased sharply during winter, cell yields were sustained. Although rates of endogenous metabolisms of 0.03 g_{cells} (g_{cells} $h)^{-1}$ for growing cultivars are too high for oligobacteria to sustain at likely rates of growth, rates for starving methanotrophs that are 30 times slower have been reported (Roslev and King 1995). Active transport costs alone are approximately 15 kJ mol^{-1} for delivery of nutrients to the cytoplasm. Resulting concentration gradients across the cytoplasmic envelope can be quite large according to measured concentrations in the metabolic pools (Button et al. 1973), engendering significant rates of leakage (van de Vossenberg et al. 1995); however, specific affinities were small enough to supply nutrients by diffusion alone. By relying on diffusion to feed the slow rates of winter-time growth, large rates of endogenous metabolisms could be mitigated.

Bacteria appear to be fully energized during summer; glucose additions had no effect on specific affinity for amino acids. However, the very low affinities for amino acids in winter are compatible with diffusion-driven transport to the cytoplasm. Medium conditioning provided by growing cultures is commonly associated with a short lag period before an inoculated population will assume its characteristic rate of growth. Most fresh water and marine species will not in fact grow from small inocula giving apparent viabilities that average about 3% of the total population, even while using modern extinction-culturing methods (Quang and Button 1998). Yet growth rates according to dilution culture (unpublished) and loss rates about equal to those for growth due to bacterivore grazing (Jochem et al. 2004) require that most are in fact growing.

5 Conclusions

Methods were developed which provided sufficient sensitivity for accurate activity measurements year-round. Careful manipulation of lakewater samples and the use of short incubation times and subsaturating concentrations provided reliable specific affinity values, which were used to examine nutrition and temperature adaptations of bacterioplankton within the bacterial flywheel. Extinction cultures were successfully employed in lakewater that have potential for further improvement in the number of species that may be successfully isolated. Yield values were resolved, which support early literature values for the radioisotope method but do not support most reported values for natural aquatic systems.

Quasi-steady state formulae and the bacterial flywheel provide a useful framework for quantifying bacterioplankton contribution to mineral cycling and adaptations to seasonal changes. In Harding Lake, contributions are a bacterial biomass that is steady, partially supported by amino acids for much of the year and ready to respond to springtime warming and substrate input. This is evidenced by seasonal biomass data, growth rate minima obtained from observed species succession, specific affinities for amino acids, and culture experiments.

Three different water types, present at different times over the annual cycle, can be described for Harding Lake. Within each water type, bacterioplankton are well-adapted to seasonal variations in substrate quality and quantity and to temperature. Adaptations to seasonal changes by bacterioplankton facilitate flywheel functioning, allowing bacterial biomass to conserve energy during periods of high energy input (summer) for use during periods of low energy input (winter). Adaptation to a narrow temperature range is evidenced by low activation energies at temperature extremes during winter and summer. High activation energies in spring enable organisms to benefit from increasing temperature and nutrient availability during that season.

A large portion of nutrition for winter organisms must be comprised of substrates other than amino acids. Data presented here show the replacement of bacterial species during the winter at a relatively constant biomass despite low specific affinities for amino

acids, and partial specific growth rates on amino acid mixture which are lower than the growth rate required for the observed species replacement. Since phytoplankton input is zero in winter, bacterioplankton must cycle its biomass with the DOC pool, which is not replenished until spring (Fig. 6). The large difference in specific affinities between winter and summer populations suggests that winter populations rely on diffusion transport and/or shift toward concurrent use of a large suite of substrate types.

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