ELSEVIER

FEMS Microbiology Ecology 17 (1995) 27–38

FEMS MICROBIOLOGY ECOLOGY

Microbial growth and resuscitation alter community structure after perturbation

Dana L. Haldeman [a], Penny S. Amy [a,*], David Ringelberg [b], David C. White [b], Rhea E. Garen [c], William C. Ghiorse [c]

[a] *Department of Biological Sciences, University of Nevada, Las Vegas, 4505 Maryland Parkway, Las Vegas, Nevada 89154-4004, USA*
[b] *Center for Environmental Biotechnology, University of Tennessee, Knoxville, Tennessee 37932-2575, USA*
[c] *Section of Microbiology, Cornell University, Ithaca, New York 14853, USA*

Received 12 September 1994; revised 4 February 1995; accepted 7 February 1995

Abstract

An increase in the number of culturable organisms and a decrease in the diversity of recoverable microbiota have been reported in deep subsurface materials after storage perturbation. The magnitude of the microbial community shift in stored samples was more pronounced at 4° C compared to −20° C. Phospholipid fatty acid analyses and acridine orange direct counts indicated that biomass did not increase significantly throughout storage. Changes in the types of fatty acid methyl esters determined over the time course indicated that some of the microbial community shift was due to bacterial proliferation. However, the recovery of new bacterial types only after the storage process suggested that some of the increase in culturable cell count was due to the resuscitation of dormant microorganisms, possibly activated by some aspect of sampling, sample handling, and/or storage. Comparison of acridine orange direct counts with phospholipid and diglyceride fatty acid content suggested that much of the biomass may have been non-living at early time points; however, after 30 days of storage most of the bacterial biomass was viable.

Keywords: Storage; Temperature; Subsurface; Growth; Resuscitation; Lipid analysis; Community structure

1. Introduction

Increases in recoverable microbiota and changes in the structure/composition of microbial communities have been reported in environmental samples after storage [1–5]. Successional changes such as these are probably ubiquitous in natural samples containing diverse microbiota, and demonstrate the importance of analyzing environmental samples as soon as possible after procurement. Zobell first described the "bottle effect" in water samples, attributing the increase in recovery of culturable cells to growth supporting nutrients concentrating on the sides of the sample containers [6]. Standard protocols for the handling of environmental samples have since been developed, and include maintenance of samples at temperatures believed to inhibit cellular proliferation without the disruption of membranes, commonly 4° C [7,8].

Interestingly, total cell counts remain relatively constant in stored subsurface samples [2–4,9], even though the numbers of culturable bacteria often increase significantly. The increase may be explained

* Corresponding author. Fax: 702-895-3956.

Federation of European Microbiological Societies.
SSDI 0168-6496(95)00008-9

in part by the proliferation of specific microbial types [3,5] with concomitant lysis of other bacterial cells. However, the recovery of bacterial types culturable only after storage, is likely due to the resuscitation of initially dormant or viable but non-culturable (VBNC) cells [5]. VBNC bacteria are present in many natural environments [10–13], and certain conditions may aid the resuscitation of these dormant bacterial cells, thereby increasing recovery. Enrichment methods for VBNC bacteria include the use of media with low nutrient concentrations [14], liquid media [11], the addition of specific metabolites [15], and osmotic or temperature shock [13,16]. Perturbations that occur during sampling and sample handling may result in such enrichment conditions. For example, temperature fluctuations, cell lysis, detachment of cells from particles, deaggregation of cells, or the physical mixing of gases, water, nutrients, or ions may play important roles in providing both adequate growth and resuscitation conditions (or, conversely, producing lethal effects) for specific microbial populations [3,5].

The purpose of the work presented here was to determine changes in the abundance and composition of microbial communities in subsurface rock, inclusive of non-culturable cells. Ester-linked polar lipid fatty acid analysis (PLFA) was used to demonstrate shifts in biomass and microbial community structure. Diglyceride fatty acid analysis (DGFA) was used as an indicator of dead biomass. Acridine orange direct counts and thymidine incorporation into DNA were used to determine bacterial numbers and growth. Diversity indices and fatty acid methyl ester analysis (FAME) were used to indicate changes in specific bacterial types that were culturable throughout storage. A second objective was to determine if storage of samples at a lower temperature, $-20°$ C, compared to the more commonly employed 4° C, would decrease the magnitude of the community shift.

2. Materials and methods

2.1. Sampling

Zeolitized rock (moisture content, 4.7%) was obtained from the wall of tunnel U12n in Rainier Mesa at the Nevada Test Site as previously described [17,18]. The depth of the sample site was approximately 400 m below the surface and approximately 2 km into the mesa. Rock was removed with flame-sterilized tools to create a sampling face approximately 25 cm into the tunnel wall. Rock was chipped from the sampling face into sterile containers, placed in coolers on ice, and transported to the laboratory where sample analysis was initiated in less than 6 h.

2.2. Sample analyses

All glassware and tools were solvent rinsed ($CHCl_3$:CH_3OH, 1:2). Approximately 5 kg of rock was ground with a sterile mortar and pestle, and was homogenized in 2qt Mason jars. Portions of homogenized rock were allocated for [^{3}H]thymidine uptake experiments, PLFA analyses and the recovery of culturable microorganisms. At specific sampling time points (Days O, 1, 3, 5, 7, 15, and 30) replicate containers stored at 4° C and $-20°$ C were sacrificed for the analyses.

2.3. Thymidine uptake

Labeled thymidine (125 nM ^{3}H-methyl-thymidine, specific activity > 64 μCi/mm activity; Amersham Corporation, Arlington Heights, IL) was added in a total of 100 μl of liquid [19] to 10 g portions of rock. Mixtures were stored at 4 and $-20°$ C in centrifuge tubes. At selected time points triplicate portions were sacrificed, the reaction was stopped and extractions were performed by the addition of NSE (0.3 M NaOH, 0.1% sodium dodecyl sulfate and 25 mM EDTA) according to Method 2 of Thorn and Ventullo [20]. Background thymidine incorporation was determined by extracting a portion of rock that was autoclaved and lysed with NSE before the addition of label.

2.4. Culturable organisms

Slurries of rock (1:10 wt/vol) were made with 0.2 μm filter-sterilized artificial pore water (APW) [21] containing 0.1% sodium pyrophosphate [17,18]. Triplicate slurries were made at 0, 3, and 30 days. Slurries were shaken for approximately 1 h to disperse bacteria and rock particles before plating in triplicate on R2A agar (Difco) [17,18]. After 2 weeks

incubation at 24° C, culturable numbers (cfu/g) and Shannon diversity indices were determined as previously described [17,18]. The five dominant organisms recovered from each time point, and both storage temperatures, were purified before analysis by FAME (Microbial ID, Inc., Newark, DE).

2.5. Acridine orange direct counts (AODC)

Duplicate subsamples (9 ml) of each pyrophosphate-diluted sample were combined with 1 ml of molten, filter-sterilized 1% Noble agar (Difco), mixed vigorously in a vortex mixer, and fixed by adding 135 μl of a 37% formaldehyde solution. Two 5-μl portions from each fixed subsample were spread evenly over 1 cm^2 circles on glass slides previously heated to 550° C (overnight) to remove residual organic material and bacteria from the glass surface. The slides were covered with an elevated Petri dish, and the smears were allowed to dry evenly for several hours. This allowed for proper adherence of the agar film to the glass surface. 100 μl of filter-sterilized 0.01% aqueous acridine orange (AO) solution was applied to the dried smears for 1 to 5 min. The stained smears were rinsed gently for 1 to 2 min with a stream of filter-sterilized distilled water. Coverslips were applied over the smears, and sealed with Vaspar. The slides were examined immediately. Counting was based on conventional epifluorescence and transmitted illumination using a Zeiss LSM-10 laser scanning microscope (LSM) fitted with a 50 W mercury arc epi-illuminator, a 100× (N.A. 1.4) phase contrast oil immersion objective lens, and 10× wide-field eyepieces. A computer-controlled, motorized x-y stage was used to facilitate counts of large numbers of fields. The number of AO-stained cells were counted in a predetermined number of microscope fields which depended on cell density in the samples [22]. A minimum of 50 standard fields were counted in each smear for high precision. A standard field was defined as an area of 1.29×10^4 μm^2 delimited by a rectangular photoreticle mounted in one eyepiece. The mean AODC ($\pm$S.D.) gww^{-1} was calculated for each time point by applying appropriate conversion and dilution factors and pooling the counts from 8 individual smears made from the duplicate subsamples.

During counting, bacterial cells were distinguished from other bacteria-sized fluorescent particles by viewing them on a Sony high resolution color monitor linked through a Nikon variable intermediate lens system set at 2× magnification to an Optronics CCD color video camera mounted on the camera port of the LSM-10. If necessary, the same cell or particle could be viewed at higher magnifications and resolution using the laser scanning mode to view laser scanned fluorescence and phase constrast images produced in the LSM [23]. The principal criteria used to identify bacterial cells were: (1) size and shape in epifluorescence and phase contrast images; and (2) fluorescence fading characteristics relative to the known behavior of AO when it stains bacteria cells. A background control count showed that the glass slide surfaces and pyrophosphate and agar solutions could have contributed the equivalent of $7.05 (\pm 0.29) \times 10^5$ green fluorescent bacteria-like particles per gww^{-1} to the AODC of each smear. This background number was deducted from the mean AODC value of each smear to arrive at a corrected mean $AODC^*$ value for each time point.

2.6. PLFA and DGFA analyses

PLFA analyses were performed as described in Kieft et al. [25], except a 2% formalin solution (30 ml per 75 g rock) was added to preserve samples before extraction rather than preservation by freezing. Duplicate samples were analyzed at 0, 3, and 30 days, with single samples analyzed at 1 and 15 days. The lipids were extracted in chloroform, methanol, and phosphate buffer as developed by Bligh and Dyer [26] and modified by White et al. [27]. Polar lipids were separated on a silicic acid column and subjected to mild alkaline methanolysis [27]. Methyl esters were separated, quantified, and identified by capillary gas chromatography/mass spectrometry (GC/MS).

Diglyceride fatty acids (DGFA) were isolated from the chloroform fraction eluted from the silicic acid column using thin-layer chromatography (60A, 250 mm thickness, Whatman, Clifton, NJ). The plate was developed in hexane:diethyl ether (80:20, v:v) and a band just above the origin (corresponding to the elution of a standard, 1, 2-dipalmitoyl-sn-glycerol, Sigma Chemical Co., St. Louis, MO) was discarded.

Diglycerides fatty acids were eluted in 5 ml $CHCl_3$:MeOH (1:1, v:v). Diglyceride fatty acid were recovered and identified in the same manner as were the polar-lipid fatty acids described above.

3. Results

3.1. Abundance

In samples stored at 4° C, the numbers of culturable bacteria increased several orders of magnitude ($5.18\ (\pm 4.96) \times 10^4$ to $3.91\ (\pm 0.72) \times 10^6$ cfu gww^{-1}) by 30 days. In the −20° C samples, the number remained relatively constant at approximately 10^4 cfu gww^{-1} over the same time period (Fig. 1.). PLFA biomass, which includes both culturable and viable-but-non-culturable cells, demonstrated an increasing trend in the 4° C samples (Fig. 2). Conversely, DGFA biomass, an estimate of non-viable cells, decreased from day 15 to 30 days of storage in the 4° C samples (Fig. 2). PLFA and DGFA concentrations in samples stored at −20° C did not demonstrate any consistent trend throughout storage.

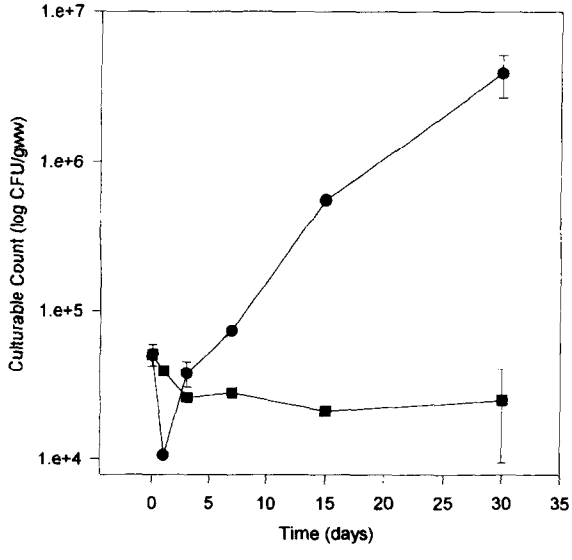

Fig. 1. Culturable bacterial counts throughout 30 days of storage at 4° C (●) and −20° C (■). Triplicate samples were analyzed at 0, 3, and 30 days. Error bars are shown for these points but may fall within the symbol, e.g. 3 days for −20° C samples.

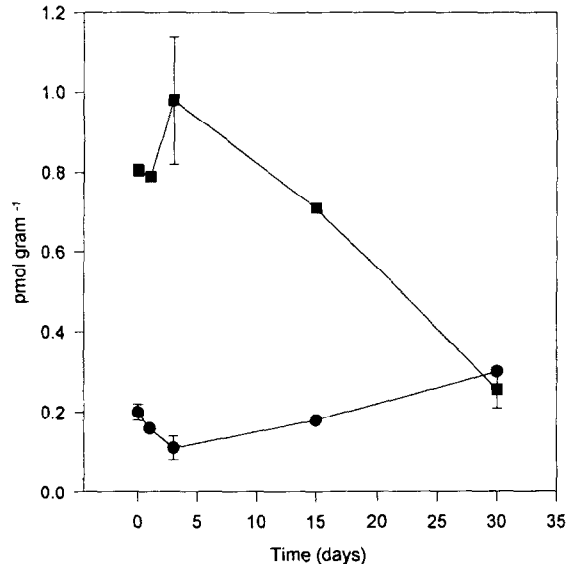

Fig. 2. Changes in total PLFA (●) and DGFA (■) content of samples throughout 30 days of storage at 4° C based on pmol per g wet weight of rock. Error bars are shown for replicate samples at 0, 3 and 30 days.

Total bacterial numbers determined by the AODC method remained within a statistically indistinguishable range of mean values throughout the storage period. The background-corrected AODC value, AODC*, ranged from 1.61×10^6 (± 0.78) to $3.08\ (\pm 1.68) \times 10^6$ cells gww^{-1}. Pooled *t*-tests showed that only the means of the samples stored for 30 days (AODC = $3.08\ (\pm 1.68) \times 10^6$ cells gww^{-1} at 4° C versus $1.67\ (\pm 0.75) \times 10^6$ cells gww^{-1}, at −20° C) were significantly different at an α level of 0.05 ($P = 0.048$); neither 30-day mean was signifcantly different from the mean of any other time point at the same α level. The mean AODC* of all time points pooled together was 2.18 (± 0.56) ($n = 6$) $\times 10^6$ cells gww^{-1}. During counting, mostly short rods and small coccoid cells, 0.5 to 1.6 μm in size, were observed. The size and shape of the cells in all samples remained relatively uniform throughout the storage period.

The rate of incorporation of [^{3}H]thymidine into DNA did not change significantly; i.e. values at all time points at both temperatures were not significantly above background controls (data not shown)

indicating that no significant bacteria growth occurred during storage.

3.2. Composition

Recovery of PLFA from the −20° C stored samples (Fig. 3B) demonstrated no consistent trend with respect to PLFA type over the time course of storage, while those recovered from the 4° C samples (Fig. 3A) remained constant until 30 days, when an increasing trend was observed in the mol percent of terminally branched saturated and monoenoic fatty acids. Increases in terminally branched saturated fatty acids were greater for the anteiso configuration which is indicative of high G + C Gram-positive organisms [28]. The increase in monoenoic fatty acids, specifically 16:1w7c and 18:1w7c, can be attributed to Gram-negative microorganisms [28]. Diglyceride-associated, terminally-branched, saturated fatty acids decreased in concentration after 30 days of storage in the 4° C samples as compared to the early time points (data not shown). This was opposite to the trend observed with the polar lipid-associated, terminally-branched saturated fatty acids.

A principle components analysis (Ensight software package, Infometrix, Seattle, WA) of the fatty acid profiles is presented in Fig. 4A. The 16:0 and 18:0 normal saturates were left out of the analysis in order to minimize any biomass effect. These two fatty acids are found in abundance in most cells, and their inclusion in the analysis would have weighted the clusters primarily by biomass as opposed to fatty acid composition. The plot illustrates that most of the samples showed little variance between the PLFA mol percent profiles. Three samples from 30-day storage (both 4 and −20° C) and one −20° C sample stored for 1 day were weighted most positively indicating the presence of different PLFA mol% profiles. A two-dimensional plot of the Eigenvector loadings (Fig. 4B) indicates that the variables a15:0 and 18:1w7c carried the greatest positive weight within the first principle component in explaining the variance between the PLFA profiles of these samples compared to the others. The PLFAs 16:w7c and 10me16:0 were assigned the greatest positive weight within principle component two, explaining the variance in the PLFA profile of the −20° C 1 day sample as compared to the others. The first and second principle components accounted for 82% and 7% of the sample variance, respectively.

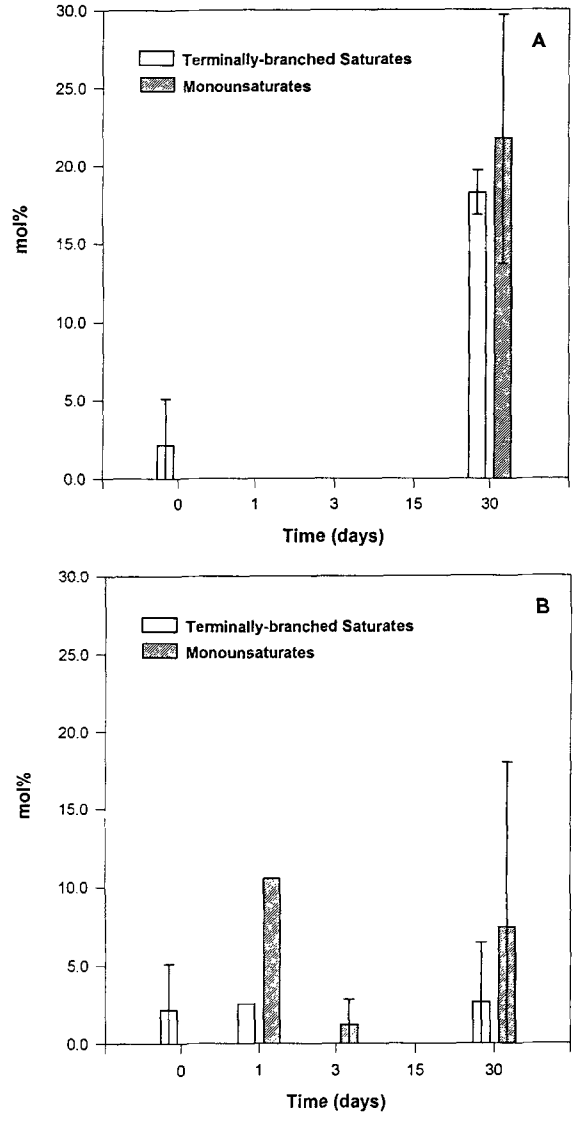

Fig. 3. Changes in specific PLFA throughout 30 days of storage at 4° C (**A**) and −20° C (**B**) based on mol percent recovery. Open bars = terminally branched saturates and shaded bars = monoenoic fatty acids.

3.3. Specific isolates

The diversity of recoverable microbiota after storage was greater in the −20° C samples as compared

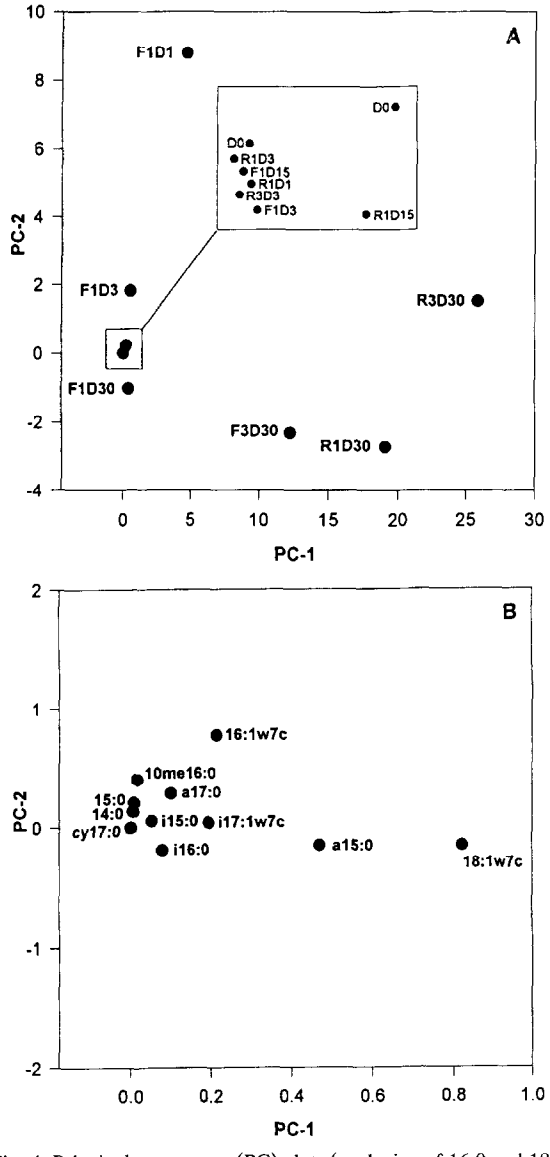

Fig. 4. Principal component (PC) plots (exclusive of 16:0 and 18:0 fatty acids) demonstrating the relatedness of samples (Panel **A**) and significance of the specific fatty acids in explaining the variance between sample PLFA profiles (Panel **B**) throughout 30 days of storage at 4° C and −20° C. Sample designations R1 and R3 (4° C) and F1 and F3 (−20° C) are followed by the letter D and the day of storage. Time zero samples are represented by D0.

to the 4° C samples (Fig. 5). The diversity of the 4° C samples continued to decrease up to 15 days of storage, but increased by 30 days. The samples stored at −20° C demonstrated a gradual increase in diversity.

Of the 105 dominant isolates that were selected for FAME analysis, 53 proved to be unsuitable for analysis by MIDI because they failed to produce sufficient biomass under the standard growth conditions specified by the manufacturer (growth on trypticase soy agar, 24 h at 28° C). Sixty percent (36/60) of the isolates recovered from the −20° C samples and 42% (25/60) of the 4° C isolates were unsuitable for testing. Of the isolates which were analyzed, a few were identified by comparison to the MIDI data base (aerobe TSBA version 3.7) at a level ≥ 0.300; a level considered acceptable for the identification of environmental organisms [18]. Of the isolates that were matched to the MIDI database, one isolate recovered from a −20° C sample stored for 30 days was identified as *Arthrobacter globiformis*. Isolates recovered from 4° C samples were identified as *Micrococcus luteus* (four isolates, one each from 7 and 15 days, and two isolates at 30 days), *Arthrobacter auerescens* (three isolates, one each at 3, 7, and 30 days), and *Staphylococcus haemolyticus* (one isolate at 7 days). The similarity coefficient for the latter isolate was not stringent enough for clinical identification of this species (≥ 0.600), although the isolate is probably adequately identified to the genus/family level.

Principle components analyses of the FAME profiles recovered from these isolates are depicted in Figs. 6 and 7. Isolates recovered from samples stored at 4° C clustered into three broad groups, one containing isolates recovered only at or before 3 days of storage (two isolates), one containing isolates predominately recovered at or after 7 days of storage (21 isolates), and one containing isolates recovered throughout storage (22 isolates) (Fig. 6A). Isolates recovered from the samples stored at −20° C clustered into three groups, all of which contained isolates recovered throughout storage (Fig. 6B). A principle components plot of the FAME profiles of specific isolates recovered from both the 4 and −20° C samples is shown in Fig. 7. While three cluster groups contained organisms isolated from both 4 and −20° C samples, a fourth cluster group contained predominantly 4° C isolates. One of the groups containing isolates from both storage temperatures clustered with *Acinetobacter* and *Bacillus*, one cluster contained 5 isolates related to *Pseudomonas*, and the largest group of isolates (33 strains)

were not matched to bacterial genera within the MIDI data base. In an *Arthrobacter* group, 21 out of the 25 isolates were recovered from the 4° C samples.

4. Discussion

4.1. Abundance

Culturable organisms increased in abundance from 10^4 to 10^6 cells gww^{-1}, after 30 days in samples that were stored at 4° C. Similar increases have been reported in samples from various subsurface materials [1–5]. Changes occurred in less than 24 h in some samples [1,4] and continued beyond 45 days in others [2,5]. Terminally-branched saturated and mono-unsaturated fatty acids increased in mol percentage through 30 days in the 4° C samples, and provided evidence for bacterial proliferation (Fig. 3A).

Although PLFA biomass measurements demonstrated an increasing trend in the 4° C samples, they were near the limit of detection. Acridine orange direct counts also were close to the limit of detection; however, the AODC method we employed is reliable in the 10^6 range [24]. Additionally, low numbers of cells may have prevented the detection of bacterial proliferation by thymidine incorporation into nucleic acids. Positive controls for thymidine incorporation contained 10^7 actively growing cells gww^{-1} and resulted in values barely above background (data not shown).

Lower conversion factors may be necessary to compare PLFA recovery to actual cell numbers in deep subsurface materials. A conversion factor of 2.11×10^4 cells $pmol^{-1}$ PLFA was calculated from data published by Balkwill et al. [29] to convert picomoles of PLFA recovered from deep subsurface material to cell numbers. This value was lower than those calculated from surface microbiota or pure cultures. Utilizing this conversion factor, numbers of total cells in this study remained relatively constant at approximately 1.75×10^3 to 1.62×10^4 cells gww^{-1} of material. The number of total cells determined by acridine orange direct counting and cfu at 15 and 30 days at 4° C, were higher in these samples, at approximately 2×10^6 to 5×10^6 cells gww^{-1} of rock. One explanation for the incongruence may be attributed to the different bacterial communities that were present in the environments sampled. Although Balkwill et al. [29] suggested that the bacteria examined in their study of subsurface aquifer material were starved, sedimentary material is more transmissive and the resident microbes would experience greater nutrient flux than in zeolitized ashfall tuff. The bacteria entrained within zeolitized rock of Rainier Mesa are believed to have been isolated for extensive periods of time, and to have experienced extreme nutrient limitation due to negligible water movement [21,30]. Therefore, the bacteria observed in this study may have been older, dormant and ultramicrocells. Further, many cells from this environment do not increase in cell size under growth conditions (unpublished data).

Another explanation for the incongruence between direct counts and PLFA biomass determinations may be attributed to the presence of a large number of non-viable cells. Unlike more dynamic environments, the zeolitized tuff of Rainier Mesa has experienced little or no nutrient flux for an extended time (age of pore water estimated to be 10–11 million years [21]) and thus, may contain a large proportion of non-viable cells. DGFA data support this hypothesis.

DGFAs are believed to be the result of phosphatase activity on membrane phospholipids shortly after cell death. As such, they may provide a means of measuring dead biomass. In this study, acridine orange direct counts remained constant throughout storage at 4° C. At the same time, culturable counts in the same samples significantly increased. This incongruence implies that organism proliferation occurred at the expense of other cells, i.e. some microbial types may have lysed due to aspects of sampling or sample handling and provided nutrients for the growth of 'weed' organisms. DGFA data support this assertion because the total concentration of DGFA (dead biomass) decreased with storage time. A concomitant increase in PLFA (viable biomass) was observed (Fig. 2). Further, opposite trends in the mol percent recovery of terminally-branched, saturated fatty acids were observed, and indicated that Gram-positive cells had the most active turnover.

PLFA measurement has been successfully correlated with other measures of biomass including total

counts, adenosine triphosphate and muramic acid content in deep subsurface environments [29]. The lower PLFA per cell ratio observed here indicates the importance of determining conversion factors appropriate for specific environments. Likewise, the measurement of DGFA may be important in determining biomass, especially in environments where large numbers of dead cells may be present.

4.2. Composition

The composition of PLFAs and culturable bacteria in this study shifted throughout storage as has been reported previously [3–5]. Although the culturable cell number represented a small proportion of the total cell number (except in samples stored for 15 and 30 days at 4° C), community structural changes were indicated. Differences were noted in the specific types of culturable microbiota, and the types and amount of PLFA recovered. Signature fatty acids for high G + C Gram-positive and Gram-negative organisms increased after 30 days of storage in the 4° C samples, as evidenced by increases in the terminally-branched, saturated and mono-unsaturated FAME, respectively (Fig. 3). A principle component analysis (Fig. 4) supports the idea that a major community shift had occurred by 30 days of sample storage.

The types of isolates that were recovered as dominant bacteria after sample storage paralleled the increases observed in some fatty acids; i.e. high G + C Gram-positive organisms (*Arthrobacter*) were the predominant culturable organisms after 30 days of sample storage, and the concomitant increase in the terminally-branched, saturated fatty acids is indicative of this bacterial group. By 30 days of storage at 4° C, the culturable cell count approached the direct cell count suggesting that nearly all cells were culturable at this time.

Diversity indices also demonstrated a community shift, but did not reveal the full extent of change. Principle component analyses of the FAME profiles of dominant isolates (Fig. 6A and B) demonstrated that different types of bacteria were recovered throughout storage while diversity indices may have been similar. For example, diversity indices at time zero and 30 days were similar (Fig. 5), while FAME profiles of dominant isolates demonstrated that the culturable bacteria were different at those two times.

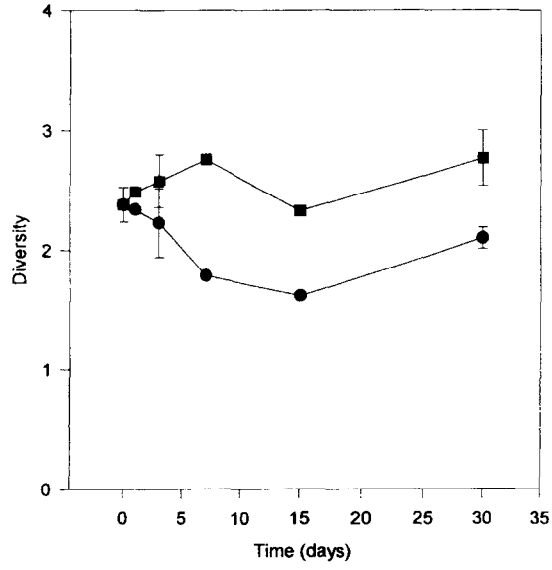

Fig. 5. Changes in diversity as indicated by Shannon diversity index throughout 30 days of storage at 4° C (●) and −20° C (■). Error bars are shown with mean diversity for triplicate samples analyzed at 0, 3 and 30 days.

4.3. Resuscitation

Resuscitation of VBNC bacteria may explain the recovery of new bacterial types throughout storage. Over 50% of all bacterial types isolated have been reported to be recovered only after storage [4,5], a phenomenon which occurred in this study as well (data not shown). Proliferation alone cannot explain the presence of these organisms after storage; therefore, they must have been resuscitated [3,5]. Preliminary results of growth experiments at 4° C, indicate that some isolates recovered only after storage double at rates which cannot account for the numbers of these cells after storage. Others have suggested that temperature, nutrient level and osmotic changes were responsible for the increased culturability of VNBC cells [13–16,30]. In this study, an entire cluster of organisms related to *Arthrobacter* was cultured only after storage at 4° C (Fig. 6A). Therefore, temperature is likely to have played an important role in the resuscitation of these organisms. Further, the numbers of new microbial types ('A' organisms in Fig.

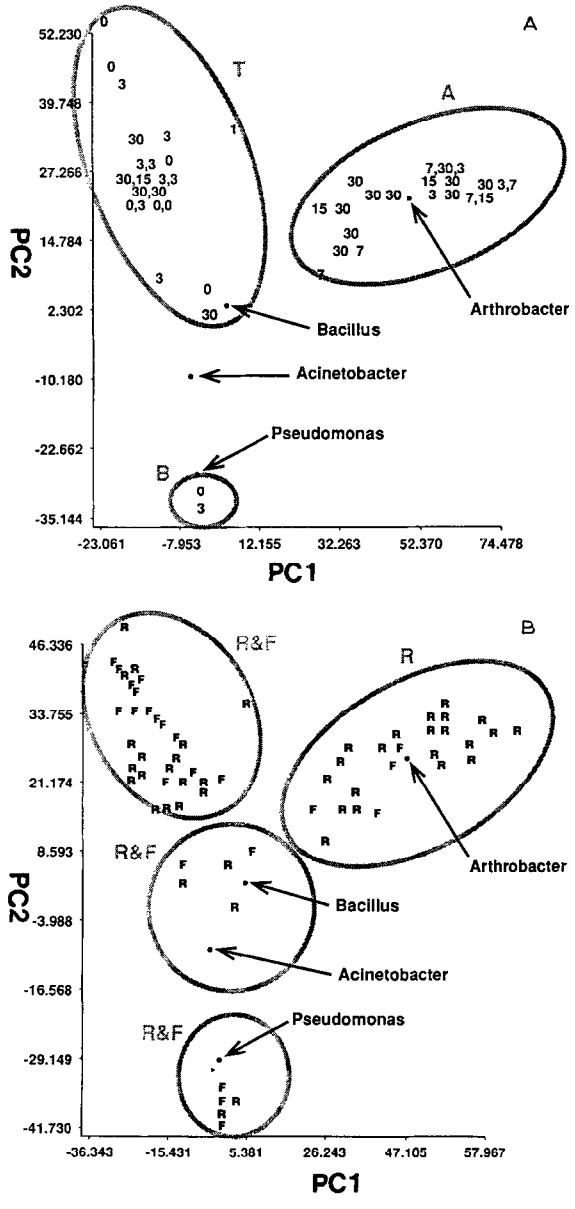

Fig. 6. Principal components plots generated from the FAME profiles of the five dominant isolates recovered from all time points and replicates at 4° C (Panel **A**) and −20° C (Panel **B**). The numbers represent the day of recovery of the individual isolates. Labelled genera are included as reference points. Circled groups contain isolates recovered predominately before (B), after (A) or throughout (T) storage.

6A) were present in great abundance in samples stored at 4° C versus −20° C. This suggests that the more moderate temperature may support the recovery of increased numbers of VBNC. Perhaps increased water availability in the 4° C samples (liquid as compared to frozen in the −20° C samples) aided in the distribution of nutrients, ions, etc., that may have also been crucial in the resuscitation of dormant bacteria.

4.4. Temperature of storage

The abundance of culturable microorganisms increased in volcanic rock that was stored at 4° C, but numbers did not appear to increase in samples stored at −20° C. Interestingly, the numbers of culturable microorganisms did not appear to decrease with storage at the colder temperature either. Loss of viability at −20° C may have been expected as freezing temperatures can disrupt cellular membranes. Lack of moisture in this rock (4.7%) may have protected bacterial cells from freezing damage. *Arthrobacter* spp. and related bacteria were commonly recovered from both sample sets after storage. *Arthrobacter* spp. have been recovered from cold antarctic environments [31] and have been shown to withstand freezing and thawing under a variety of laboratory conditions [32].

Previous research has shown that a more rapid change in microbial communities occurs in deep subsurface samples that were stored at warmer temperatures [4], while no increase in culturable count was observed throughout 30 days of storage at −20° C. Although it is tempting to suggest that materials for microbiological analysis be stored at −20° C because microbial diversity was maintained throughout storage (Fig. 5), it should be noted that the communities recovered from the different temperature treatments were not identical (Fig. 7). Three of the four clusters contained isolates from both temperature treatments, but one very large cluster contained isolates that were only recovered from refrigerated samples.

The variability observed in proportions of specific fatty acids in frozen samples (Fig. 3B), especially the PLFA concentration after one day of storage, cannot be explained. If it were due solely to the natural heterogeneity of the rock, similar effects would likely have been observed in the refrigerated samples as well. All rock was homogenized before separation into the two temperature treatments.

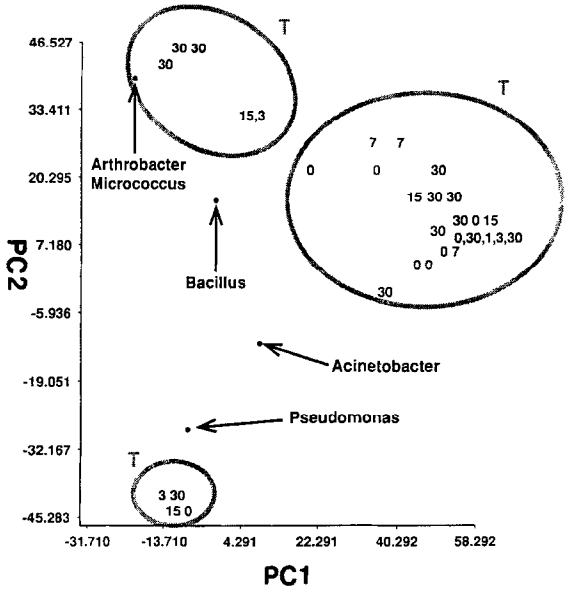

Fig. 7. Principal components plots generated from FAME profiles of the five dominant isolates recovered from all time points (including all replicates) at 4° C (R) and −20° C (F). Circled groups contain isolates recovered predominately from 4° C samples (R) or both temperatures (R and F).

4.5. Conclusions

Sampling and sample handling may provide the stimuli necessary for the growth and resuscitation of some bacterial types as evidenced by change in numbers of culturable cells, cell types, and types of lipids recovered throughout storage. The recovery of specific bacterial types varied at sub-zero temperatures, as well, even though culturable cell counts did not diminish. Total cell counts remained relatively constant at both storage temperatures and when compared with PLFA and DGFA analyses, stress the importance of these measurements, particularly in environments where cells are likely to be dead or dormant, e.g. the deep subsurface.

Acknowledgements

We thank Dr. Bob Griffiths, Beth Pitonzo, Kathy Davidson, Eric Anderson, Bill Petroutsen, Chuck Russell and Rosamond Thomas for assistance in sampling and Tina Richey for MIDI analyses. This work was supported by the U.S. Department of Energy grants DE-UNLV-60975, #DE-FG05-90ER60988, and a Nevada DOE EPSCoR traineeship grant.

References

[1] Hirsch, P. and Rades-Rohkohl, E. (1988) Some special problems in the determination of viable counts of groundwater microorganisms. Microb. Ecol. 16, 99–113.
[2] Brockman, F.J., Kieft, T.L., Frederickson, J.K., Bjornstad, B.N., Li, S.W., Spangenburg, W. and Long, P.E. (1992) Microbiology of vadose zone paleosols in south-central Washington state. Microb. Ecol. 23, 279–301.
[3] Haldeman, D.L., Amy, P.S., White, D.C. and Ringelberg, D.B. (1994) Changes in bacteria recoverable from subsurface volcanic rock samples during storage at 4° C. Appl. Environ. Microbiol. 60, 2697–2703.
[4] Fredrickson, J.K., Li, S.W., Brockman, F.J., Haldeman, D.L., Amy, P.S. and Balkwill, D.L. (1995) Time-dependent changes in viable count and activities of heterotrophic bacteria in subsurface samples. J. Microbiol. Meth. 21, 253–265.
[5] Haldeman, D.L., Amy, P.S., Russell, C.E. and Jacobson, R. (1995) Comparison of drilling and mining as methods for obtaining microbiological samples from the deep subsurface. J. Microbiol. Meth. 21, 305–316.
[6] Zobell, C.E. (1943) The effects of solid surfaces upon bacterial activity. J. Bacteriol. 46, 39–56.
[7] Atlas, R.M. and Bartha, R. (1987) Microbial Ecology: Fundamentals and Applications, 2nd Edn. Benjamin/Cummings Publishing Co., Inc. Menlo Park, CA.
[8] Wollum, A.G. (1982) Cultural methods for soil microorganisms. In: Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties. (Page, A.L., Ed.) pp. 781–802, American Society of Agronomy, Madison, WI.
[9] Ghiorse, W.C. and Balkwill, D.L. (1985) Microbiological characterization of subsurface environments. In: Ground Water Quality. (Ward, C.H., Giger, W. and McCarty, P.L., Eds.) pp. 387–401, Wiley, New York.
[10] Rozak, D.B. and Colwell, R.R. (1987) Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51, 365–379.
[11] Kapreylants, A.S. and Kell, D.B. (1993) Dormancy in stationary-phase cultures of *Micrococcus luteus*: Flow cytometric analysis of starvation and resuscitation. Appl. Environ. Microbiol. 59, 3187–3196.
[12] Bissonette, J.K., Jezeski, J.H., McFeters, G.A. and Stuart, D.G. (1975) Influence of environmental stress on enumeration of indicator bacteria from natural water. Appl. Environ. Microbiol. 29, 186–194.
[13] Nilsson, L.L., Oliver, J.D. and Kjelleberg, S. (1991) Resuscitation of *Vibrio vulnificus* from the viable but non culturable state. J. Bacteriol. 173, 5054–5059.
[14] Caldwell, B.A., Ye., D., Griffiths, R.P., Moyer, C.L. and Morita, R.Y. (1989) Plasmid expression and maintenance

during long-term starvation-survival of bacteria in well water. Appl. Environ. Microbiol. 55, 1860–1864.
[15] Henis, Y. (1987) Survival and dormancy of bacteria. In: Survival and Dormancy of Microorganisms (Henis, Y., Ed.) pp. 1–108, Wiley, New York.
[16] Murno, P.M., Gauthier, M.J., Greittmayer, V.A. and Bongiovanni, J. (1989) Influence of osmoregulation processes on starvation survival of *Escherichia coli* in seawater. Appl. Environ. Microbiol. 55, 2017–2024.
[17] Haldeman, D.L. and Amy, P.S. (1993) Bacterial heterogeneity in deep subsurface tunnels at Rainier Mesa, Nevada Test Site., Microb. Ecol. 25, 183–194.
[18] Haldeman, D.L., Amy, P.S., Ringelberg, D. and White, D.C. (1993) Characterization of the microbiology within a 21 m^3 section of rock from the deep subsurface. Microb. Ecol. 26, 145–159.
[19] Kaplan, L.A., Bott, T.L. and Bielicki, J.K. (1992) Assessment of [^{3}H]thymidine incorporation into DNA as a method to determine bacterial productivity in stream bed sediments. Appl. Environ. Microbiol. 58, 3614–3621.
[20] Thorn, P.M. and Ventullo, R.M. (1988) Measurement of bacterial growth rates in subsurface sediments using the incorporation of tritiated thymidine into DNA. Microb. Ecol. 16, 3–16.
[21] Amy, P.S., Haldeman, D.L., Ringelberg, D., Hall, D.H. and Russell, C. (1992) Comparison of identification systems for classification of bacteria isolated from water and endolithic habitats within the deep subsurface. Appl. Environ. Microbiol. 58, 3367–3373.
[22] Beloin, R.M. (1987) Master's Thesis. Cornell University, Ithaca, NY. Thesis (p. 7).
[23] Ghiorse, W.C., Miller, D.N., Sandoli, R.L. and Siering, P.L. (1995) Application of laser scanning microscopy for analysis of aquatic microhabitats. In Fred Dobbs (Guest Editor). Special Issue on Microscopic Studies of Microorganisms (manuscript submitted).
[24] Reference omitted.
[25] Kieft, T.L., Ringelberg, D.B. and White, D.C. (1994) Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and dessication in a porous medium. Appl. Environ. Microbiol., in press.
[26] Bligh, E.G. and Dyer, W.M. (1959) A rapid method of lipid extraction and purification. Can. J. Biochem. Physiol. 35, 911–917.
[27] White, D.C., Davis, W.M., Nickels, J.S., King, J.D. and Bobbie, R.J. (1979) Determination of sedimentary microbial biomass by extractable lipid phosphate. Oecologia. 40, 51–62.
[28] O'Leary, W.M. and Wilkinson, S.G. (1988) Microbial Lipids, Vol. 1. (Ratledge, E. and Wilkinson, S.G., Eds.) p. 122. Academic Press, London.
[29] Balkwill, D.L., Leach, F.R., Wilson, J.T., McNabb, J.F. and White, D.C. (1988) Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments. Microb. Ecol. 16, 73–84.
[30] Amy, P.S., Durham, C.D., Hall, D. and Haldeman, D.L. (1993) Starvation survival of deep subsurface isolates. Curr. Microbiol. 26, 345–352.
[31] Shivaji, S., Shymala Rao, N., Saisree, L., Reddy, G.S.N., Seshu Kumar, G. and Bhargava, P.M. (1989) Isolates of Arthrobacter from soils of Schirmacher Oasis, Antarctica. Polar Biol. 10, 225–229.
[32] Nelson, L.M. and Parkinson, D. (1978) Effect of freezing and thawing on survival of three bacterial isolates from an antarctic soil. Can. J. Microbiol. 24, 1468–1474.