Temperature effects on Biolog community level physiological profiles

by

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ABSTRACT

The effect of low incubation temperatures on Biolog Community Level Physiological Profiles (CLPP) was investigated by performing CLPPs at 6° and 15°C. Samples were obtained from the Cornwallis River (45° 05'N, 63° 35'W) and standardized to an initial density of approximately 10^6 cells/ml (AODC). A preliminary comparison of GN plates with SFN plates (which lack tetrazolium dye) confirmed that the dye chemistry worked at low temperature. Various reference points from Adjusted Optical Density (AOD) and Average Well Colour Development (AWCD) values were calculated for each plate in addition to kinetic parameters of lag and slope. Responses at 6°C were predictably slower than those at 15°C, but most eventually reached comparable asymptotes. Principal Component Analysis (PCA), followed by ANOVA of the PCA scores, revealed that the 6°C responses were not significantly different on all AOD parameters. Another PCA suggested that AWCD 0.5 represents a profile due to a change in the bacterial community of the plate over time, and not an intrinsic difference in the community patterns due to incubation temperature. Given that the initial community structure was identical for each temperature, this implies that AWCD 0.5 picks up enrichment that took place in the wells. This measurement is likely too late to detect true metabolic diversity of the community before sample enrichment has occurred. Lag, an early measurement, may reveal subtle community differences prior to sample enrichment. Slope is not favourable when measuring temperature effects on CLPPs, as this parameter emphasizes the large differences in rate of colour development simply due to thermodynamics. Additional information was gathered on the thermodynamic effects of substrates using Arrhenius plots. Similar experiments were performed to compare summer and winter samples incubated at 15°C. AOD, AWCD and kinetic parameters revealed that there were no community differences in river samples collected during summer versus winter months. Identification profiles from river isolates confirmed that the river bacterial community is not significantly altered with environmental temperature changes.

GENERAL INTRODUCTION

Rivers are very important to the subsistence of most living organisms. Obvious value is often placed on their source of food and their use for the irrigation of agricultural crops. The Annapolis Valley of Nova Scotia is a prime example of this. The Cornwallis River, in particular, is one of the primary sources of water used by farmers and other industries located along its course. It is 40 km long and empties into the Minas Basin of the Bay of Fundy. The river is a source of essential water for locals, but unfortunately, the Cornwallis River also receives some municipal, agricultural, and food processing wastes as it empties into the estuary. Sewage treatment facilities, fruit and vegetable canneries, a meat packing plant, and poultry processing plants are some of the contributing sources of wastewater loading into the river.

The biological functions of bacterial communities can be estimated by the measurement of their microbial diversity. Environment changes lead to changes in the community structure, and so, as the community changes, so does its diversity community (1). Bacterial communities must be kept intact to most accurately replicate their environment. The Biolog assay has been used as a tool to measure the metabolic potential of microbial communities typically using whole terrestrial and aquatic samples. In this study, the use of Biolog was tested at a lowered incubation temperature using water samples from the Cornwallis River. Its potential to measure community changes and provide more insight on the ecology of any habitat was also examined.

Community Level Physiological Profiles

Community Level Physiological Profiles (CLPP) were first introduced in 1991 by Garland and Mills (7) as a rapid method for characterizing microbial communities based on inoculation of whole community samples into Biolog 96 well microtitre plates. The Biolog assay has become popular because of its simplicity and rapidity. It measures carbon utilization via the reduction of tetrazolium dye, which yields the production of colour. This colour/turbidity is then measured using a plate reader by taking the optical density of each well. Originally, the Biolog system was introduced as a method to identify pure microbial cultures. The profile obtained from the 95 carbon sources (95 carbon substrate wells plus a control well which contains only tetrazolium dye) following an incubation period is matched with a distinct pattern from a known bacterial species found in Biolog's database.

Although the method involved in characterizing/measuring whole bacterial communities is rapid and simple, understanding what is being measured and what approach should be taken is much more complex than it appears. Since its "re-introduction" as a novel way for microbial ecologists to investigate bacterial communities, several studies have examined inoculum density (7, 30) reproducibility of profiles (11), plate incubation period (33), and statistical techniques (8, 12, 14) to better understand its application and the interpretation of results obtained using this whole community approach. All have the same goal: to develop a reliable and practical method to analyze the information provided by this approach.

Inoculum density

There already have been several attempts to standardize certain aspects of the measurement of microbial communities and the interpretation of results. For example, the importance of using a specific range of inoculum densities for all whole sample studies has been examined carefully. Garland and Mills (7) suggested that inoculum density had an effect on the optical density readings of whole environmental samples. Winding (30) then found that an increase in inoculum cell concentration decreased the lag period prior to colour development, but had no influence on the total colour intensity of the number of wells with colour development. Subsequent kinetic studies with Biolog indicated that the rate of formazan formation and the number of wells with significant colour change did increase with higher inoculum density (5, 11, 31). A concentration of cells/ml greater than 10^4 but less than 10^7 was shown to be optimal during the initial inoculation step. A subsequent minimum incubation period was required to attain sufficient cell density for colour development and growth of the initial inoculum to about 10^8 cells/ml. This concentration was necessary for the formation of formazan. Inoculation of a greater number of cells per well resulted in colouration of the control well (31). To date, equivalent cell concentrations inoculated in each plate are considered necessary for reliable comparison of bacterial communities (7, 30).

Enrichment effect

Once inoculum density is normalized, there is still the issue of the enrichment effect following the incubation of Biolog plates. It has been shown that the initial microbial community inoculated in the plates does not reflect the resulting community

structure present in the wells following incubation (6, 11, 13, 18, 24, 28). Although oxidation of the substrates depends on both the composition and the density of the inoculum used, it remains unclear what fraction of the sample community actually contributes to the pattern of substrate use. Smalla *et al.* (13) concluded that when the Biolog method was applied to samples of potato phyllosphere, the Gram positive phyllosphere bacteria rarely contributed to the community pattern. Experiments were also performed to detect bacterial community shifts from field-grown potato leaves due to incubation in Biolog GN plates. Their results indicated a reduced diversity of colony types. The number of Gram negative species decreased from thirteen to eight, and no Gram positive species could be detected at all following incubation. They urged to use caution when using Biolog for catabolic profiling due to its selectivity for fast growing heterotrophic bacterial populations.

Timing of Biolog microtitre plate readings

Caution must be used when interpreting Biolog results. The timing of reading Biolog plates is critical to their interpretation. Readings taken later in the incubation period (when wells have turned positive) reflect the dominating or enriched species present in the plate (13). Glimm *et al.* (8) found that earlier readings reveal the contribution of a more diverse community rather than later readings which are more likely to reflect the intensity of dominant species.

Several attempts have been made to find the best way to obtain information about the colour development in Biolog plates. Many studies use single point optical density readings and find the average well colour development (AWCD) of the plate, an idea

established by Garland and Mills (7). While this method is quick and simple, there is a lack of standardization between studies using this measurement. The optimum reading time varies with sample type, the temperature at which the plate is incubated, and the analysis.

Several authors have used kinetic analyses over time as a measurement of colour development to avoid some of the variability involved in bacterial community studies. Guckert *et al.* (10) calculated the area under the absorbance versus time curve for each well using a trapezoidal model. This approach is advantageous in that a single value integrates information from the entire incubation period. This value incorporates the lag phase, the rate of development and the extent of colour development for each substrate. Another approach is to curve fit each absorbance versus time plot, and calculate the lag, slope, and asymptote for each well (19, 31). Although much more labour intensive, this approach avoids having to select the most appropriate time point for measurement.

Statistical analyses

Even when clear objectives have been identified, the most appropriate statistical approach, or combination of techniques to analyze and interpret substrate utilization from Biolog data has yet to be found. Due to the very large number of variables (95 substrate utilization profiles), multivariate testing is the method of choice in the analysis of CLPPs. When the number of variables per observation is higher than the number of observations itself, then multivariate type analyses are useful and accepted. Multivariate analysis reduces the number of variables and allows significant differences between communities to be examined (25). The most popular multivariate test in Biolog studies has been

principal components analysis (PCA). While two or three-dimensional plots are the most common, they also allow community differences to be visualized most easily. Additional patterns between communities may be discovered with five principal components, but some might find this just adds confusion to the interpretation.

Project objectives

The objectives of this study were (i) to examine the effect of low incubation temperatures on Cornwallis River bacterial communities using Biolog Community Level Physiological Profiles (CLPPs), and use these effects in such a manner as to determine which parameter is best suited to measure community profiles at low temperatures, and (ii) to examine the ecology of the Cornwallis River to evaluate how Biolog could be used to detect community changes in the river.

Chapter 1: Biolog and low incubation temperatures

INTRODUCTION

Several microbial ecologists have used Biolog Community Level Physiological Profiles (CLPPs) for the past decade as a tool to study bacterial communities from a variety of habitats (5, 7, 10, 11, 19, 31). Biolog has been used with a variety of samples including, assorted soil types (24, 30), marine and freshwater habitats (2, 9), and wastewater (23, 26, 29). Using such samples, several components were identified including: inoculum density (5, 11, 30, 31), enrichment of the sample in the plate (13), and single plate readings versus the use of kinetic analyses (4, 5, 10, 17, 19, 32). One area of the Biolog assay that has yet to be examined is the potential effect of a lowered incubation temperature on CLPPs. Before such effects can be tested, a few issues must be addressed. First, is the tetrazolium dye reduced at lower temperatures? Since most of the studies have been performed using incubation temperatures of 15°C (12, 19), and up to 28°C (5, 8, 10, 13, 24), a test on the dye chemistry in Gram negative (GN) Biolog plates at a temperature lower than 15°C was required. Second, given that lower inoculum densities result in a decrease in colour development (11, 17, 27, 30, 31), could different patterns in substrate utilization due to a change in temperature be measured or detected? Lowering the incubation temperature of Biolog plates would inevitably have a similar effect to a decrease in inoculum density simply because of thermodynamics. It is known that an increase in inoculum concentration increases the rate of colour development (5, 11, 31), and shortens the lag before colour development occurs (30).

An additional parameter was later added to this study to further identify thermodynamic effects. It is referred to as the temperature characteristic. It is a measure of the energy of activation of a reaction and equals the slope of the Arrhenius curve (22). An Arrhenius plot is the logarithm of any rate plotted against the reciprocal of the absolute temperature. Temperature characteristic can be calculated from the formula:

$$\text{temperature characteristic} = \frac{2.303RT_1T_2(\log 10rate2 - \log 10rate1)}{T_2 - T_1}$$

where the rate refers to the slope parameter value calculated from each curve at absolute incubation temperatures T_1 and T_2 and R is the universal gas constant (1.98 cal/mole) (21). The greater the slope of the Arrhenius curve, the larger the temperature characteristic value. This value facilitates the identification of potential patterns in substrate utilization (kinetics) between 6°C and 15°C. The purpose of including this simple parameter allows you to check its usefulness in future studies involving substrate kinetics at different temperatures.

MATERIALS AND METHODS

Sample Collection

The sample site chosen for this study was the Cornwallis River (45° 05'N, 63° 35'W). This river is about 40 km long and flows into the Minas Basin. Samples were regularly collected beneath a highway bridge near the town of Coldbrook where the riverbanks are predominantly pastures and woodlands. Seven samples were collected weekly from February 13 and March 25, 1996. Two sterile plastic Nalgene ™ 500ml bottles were used for sample collection. The temperature and depth of the river were

recorded. All samples were immediately carried back to the laboratory on ice and processed within 2 hours.

Biolog Multisubstrate Plate Inoculation

Whole river samples were diluted (1/5) with phosphate buffered saline to bring the inoculum cell concentration to approximately 10^7 cells/ml. Samples were then inoculated into Biolog GN microtitre plates (Biolog Inc., Hayward, CA). Four plates were inoculated for every sample. Two plates were incubated at 15°C (W15), and the other two at 6°C (W6). The optical density in each well of the plates incubated at 15°C was measured every 6-8 h for 120 h. The plates incubated at 6°C were read every 24 h for a total of 336 h (14 days). The optical density was measured using the Series 750 Microplate Reader (Cambridge Technology, Inc.), in combination with the Microlog 3N 3.01A program Version DE (BIOLOG Inc., Hayward, CA).

An additional experiment was later carried out to test the effects of low incubation temperatures on the tetrazolium dye chemistry of the Biolog GN plates. A river sample from the same sampling site was collected in the month of June and was diluted (1/5) with PBS and inoculated into 3 Biolog GN plates and 3 Biolog SF-N plates. The SF-N plates are identical to the GN plates, without any tetrazolium red-ox dye present in the wells. All plates were incubated at 6°C for 14 days. The optical density in each well of each plate was measured every 24 h using the previously mentioned plate reader. Additional GN and SF-N plates were inoculated with samples taken from the same site and incubated at 25°C for 48 h. These were set up as an approach to compare the optical

density readings reached at 25°C to those of the low temperature plates following the incubation period used for all CLPPs of 14 days at 6°C.

The SF-N plates were not expected to reach the optical density levels of GN plates simply because SF-N plates lack the necessary dye for the production of colour. One would expect the measurement of colour and turbidity to exceed the readings of turbidity alone. But this test would provide enough information to determine whether the dye chemistry in GN plates was impaired at low temperatures.

Data manipulations

Each Biolog optical density (OD) reading was appended to the previous reading and stored as an ASCII file. After the readings were completed, these files were parsed and formatted so that each plate reading was arranged in a single row, consisting of 96 values (each value representing the optical density of a well). The incubation period that each plate was read was attached to the end of each row. The changes in optical density over time could then be followed more easily. The data were parsed and formatted using Quattro Pro (Borland International, Inc., Scotts Valley, CA), with the application of a macro created by Mark Dowe from the Microbial Ecology Lab at Acadia University.

The optical densities of each plate were imported into Sigma Plot for Windows Version 2.01 (Jandel Scientific Corporation, Corte Madera, CA) and plotted against time. Each graph was created using data from each of the duplicate plates, to follow the changes in optical density over time.

A transform function, MEANS.XFM (Appendix I) created in Sigma Plot was used to calculate the average optical density values of each well for the set of plates.

Vertical standard deviation error bars were used to indicate the variances in optical density for each time between the duplicate plates. The mean values were then plotted against time and a logistic function (LOGISTZ.FIT) was used produce a best-fit curve of the data (Appendix I). This logistic function also provided three mathematical parameters (a, b, and c) which define a sigmoidal growth curve (30). The parameters represent the asymptote: A, the slope: b, and the point of inflection of the curve: c, respectively. Zweitering *et al.* (34) expressed the mathematical parameters to describe growth kinetics in biological studies. The asymptote was defined as the maximal value reached, slope as the tangent to the inflection point, and the lag phase as the x-intercept of the tangent (Appendix IV). The lag phase is the amount of time elapsed between zero growth and the start of the exponential growth phase. Asymptote, slope and lag phase were calculated using the PLATES.XFM transform (Appendix I). One file containing the three biological parameters for all 96 wells of all samples was compiled and imported into SPSS (Statistical Package for the Social Sciences) (SPSS Inc., Chicago IL) for analysis. This file was arranged in columns by month, well, day, row, and detail, where each row represented one well.

The logarithm of the rate of colour development (slope parameter) was calculated for W6 and W15. The temperature characteristic of each set of points was calculated using the Arrhenius equation. The 95 temperature characteristics were then reduced to groups containing substrates with low, middle, and high temperature characteristics. Supporting data (river temperature, tritiated thymidine uptake, AODC, total and fecal coliform counts, and plate counts) was also added to this file. These results are discussed in chapter 2.

Additional columns labelled AOD25, AOD50 and AOD75 were later appended to the above-mentioned file. These represent the adjusted optical density (AOD) when 25, 50 and 75% of the wells in the Biolog plate were positive. A well is considered positive when there is a greater than 40% change in its initial optical density. A transform called PERCHANG.XFM was utilized to obtain the percent change in OD and the ADJUSTOD.XFM transform was created to subtract the control well absorbance value from the 95 substrates (Appendix I). The equation [(R-C/C) x 100-background)] took into account the background colour produced by each substrate, by subtracting this value (provided by BIOLOG Inc.) of each well from the percent change value (Appendix II). Both transforms were created in Sigma Plot.

Plots illustrating the number of positive wells versus time for W15 and W6 samples were used to calculate the times when 25, 50 and 75% of the wells were positive. A sample graph was created to illustrate how these reference times were calculated (Appendix III). These times were then used to determine the AOD at that particular time. The AOD was calculated by subtracting the raw OD of the A1 (control) well from the 95 response wells. The adjusted optical density values for each time were imported into SPSS as AOD25, AOD50, and AOD75.

The average well colour development (AWCD) is the adjusted optical density divided by 95 wells (7). This value is equivalent to the mean AOD for all 95 response wells on the plate. A middle reference point of AWCD 0.5 absorbance units and the incubation time corresponding to it was determined for comparison with AODs. The incubation periods necessary to reach readings of AOD25-75 were used to calculate corresponding AWCDs. The number of positive wells was also plotted against AWCD.

Statistical Analyses

The multivariate analysis method of choice was principal components analysis (PCA). In PCA the summary statistic for each plate (or sample) is regarded as a 95-component observation. The aim is to identify a small number of linear combinations of the 95 substrates, which account for as high a proportion of the plate-to-plate variation as possible. Prior to the analysis, the raw data is transformed to a correlation matrix and principal components are extracted using these correlations. Only two or three components are retained for each analysis. The loading or score measures the contribution of a variable to the component and these scores are then used to create a two or three-dimensional scatterplot. This type of plot illustrates the major sources of sample to sample variation. The component scores reveal which substrates contribute most to the principal components. Samples in the scatter plot are positioned so that samples with similar scores are located closer together on the plot; samples with dissimilar profiles are separated (25).

Several PCAs based on the various reading time points were conducted. Similar analyses on lag phase and slope revealed additional information in the comparison of the effects of incubation temperature (15°C versus 6°C) on these two parameters.

RESULTS

The effect of low temperature on the dye chemistry of Biolog plates

As mentioned, Biolog SF-N and GN plates are identical to each other, except that SF-N plates do not contain the tetrazolium red-ox dye present in GN plates. Since the

wells in SF-N plates cannot produce a change in colour, the OD readings from GN and SF-N plates are referred to as the average well OD rather than the standard AWCD. The GN and SF-N plates placed at 6°C were incubated for 336h (14 days). Each set produced sigmoidal curves when OD_{590} was plotted against time (FIG. 1.1). The GN and SF-N average well OD values were comparable until 240h. From 264 hours to the final reading time at 336h, the GN values increased more rapidly than the SF-N values. The GN plates reached a final average well OD of 0.9180 absorbance units, where SF-N plates reached 0.5115 absorbance units. The GN control for the experiment was incubated at 25°C for 48 hours. Biolog Inc. suggests a temperature of 30°C for 24h with pure cultures, but it was kept consistent with the recommended 25°C for 48h for SF-N plates. The GN plate at 25°C reached an average well OD of 1.0126 for a difference of 0.0946 absorbance units. Following incubation, the control SF-N sample reached a reading of 0.4467 while the SF-N test plates reached a maximum average well OD of 0.5115. In this case, a decrease in temperature resulted in an increase in OD by 0.0648 absorbance units.

Temperature and the rate of colour development

The AWCD was calculated for both W6 and W15 and plotted against time. This graph was then compared to the number of positive wells plotted against time (FIG. 1.2). The W15 samples reached an AWCD of 0.5 absorbance units after 90h, and W6 samples reached this point after 275h. For W15 samples, 85 of 96 wells were positive after an incubation period of 90h. Following a 275h incubation period, 82 of 96 wells were positive for W6 samples. Thus, even when the incubation temperature was considerably lower, the river community produced positive substrate utilization in over 80% of the

wells at an AWCD of 0.5. These two plots in figure 1.2 reveal that when a midpoint value for an entire plate such as AWCD 0.5 is used, it measures the average colour development when a large percentage of the wells have turned positive. To find the significance of both measurements with respect to each other, the two approaches were compared directly by plotting the number of positive wells for each sample week of W6 and W15 samples against the corresponding AWCD (FIG. 1.3). The times when 25%, 50%, and 75% of the wells had turned positive for all seven sample weeks were used as reference times to calculate corresponding AWCDs. There was a total of 21 points for W15 and 21 for W6 Despite the differences in incubation temperature, when 80 - 85 of the wells were positive, both W15 and W6 had a corresponding AWCD of approximately 0.2 to 0.3 absorbance units. Almost 100% of the wells for each sample type had gone positive before the plate reached an AWCD of 0.5 absorbance units. No positive wells at AWCD 0.75 absorbance units were included in the graph. This is because most wells had already gone positive or the time corresponding to AWCD 0.75 was later than the total incubation period for that particular sample. Thus, readings taken when 25, 50, and 75% of the wells were positive resulted in a shorter incubation period and a greater decrease in absorbance values than when classic AWCD reference points were used to measure substrate utilization.

Four separate PCAs were performed using AOD25, 50, 75 and AWCD 0.5. For each analysis two principal components (PC1 and PC2) were retained. The first (PC1) is the linear combination of observed variables that maximally separates samples by maximizing the variance of their component scores. The second component (PC2) is created from the variability remaining in the data set after the variance associated with

the first component is removed (25). The component scores are then used to create a scatter plot. In this study, the markers were set by incubation temperature and the scores for each component were plotted with PC1 on the y-axis and PC2 on the x-axis. W15 and W6 samples produced comparable scores along both PC1 and PC2 for AOD25 (FIG. 1.4), with the exception that two of the seven W6 samples had negative scores along PC1. These two points represent the first two weeks of sampling, February 13 and 22 (FIG. 1.4). Temperature had no significant effect on the AOD25 parameter along PC1 ($p=0.235$) and PC2 ($p=0.677$). Overall, a larger number of W6 samples were distributed towards the top with higher PC1 scores than W15 samples when using AOD50 (FIG. 1.5) and AOD75 (FIG. 1.6). But a one-way ANOVA determined that incubation temperature had no significant effect on either parameter ($p=0.094$ and $p=0.172$ respectively). The fourth PCA was done using AWCD 0.5 values (FIG. 1.7). Sample weeks 1, 2, 3, and 6 for both W15 and W6 samples were distributed towards the top of the plot with positive PC1 scores. The remaining sample weeks 4, 5, and 7 were found toward the bottom of the plot with negative PC1 scores. To check for the significance of these evident groups, a one-way ANOVA on the AWCD 0.5 PC1 scores revealed that temperature was not responsible for the separation of these particular samples ($p=0.642$). Another one-way ANOVA confirmed that the week of sampling had significantly affected the AWCD 0.5 parameter ($p=0.001$). Along PC2 there was a definite grouping of samples by incubation temperature. The W6 samples were slightly scattered but all had negative PC2 scores. The W15 samples, with positive PC2 scores were positioned to the right of the figure. The effect of incubation temperature on the AWCD 0.5 parameter was significant

(p=0.001) along PC2. This axis accounted for 15.1% of the variance within the component scores.

Sensitivity of substrates to low temperature

The logarithm of the slope of each curve for both W15 and W6 were placed into three separate groups and plotted against each other. The line joining the points was labelled as the temperature characteristic. If the slope of the line joining each point resulted in a high, middle, or low value, then the substrates belonging to these groups were given these labels (FIG. 1.8). Twenty-six substrates were placed in the low temperature characteristic group, 53 in the middle group, and 11 in the high group. If a substrate used by W6 samples demonstrated little colour development, then the slope value of that particular substrate curve was low. If the same well showed greater colour development for W15 samples, then the slope of the line between W15 and W6 samples was high. In this case a high temperature characteristic value indicates little carbon utilization at 6°C for that substrate. The substrates belonging to the high group are listed in Table 1. The temperature characteristic values for the low and middle groups are not listed but averaged between 16000 (low) to 23000 (middle). Five substrates consistently had slope values of zero, resulting in a temperature characteristic value of zero as well. Four of them are carboxylic acids, and one is an amino acid. These substrates were not included in the analysis and are listed in Table 2.

Kinetic parameters

The asymptote, lag, and slope were also calculated for each logistic curve. Some curves did not level off within the incubation period. These curves had asymptote values which were unrealistically high and not representative of the upper absorbance values of those particular wells. For these reasons, the asymptote was not used in any of the analyses to discriminate sample profiles.

Not surprisingly, a lower incubation temperature resulted in a longer lag period. The colour development curves for W6 had much longer lag periods than W15. A graph with the mean lag values per week was plotted for the W15 and W6 samples (FIG. 1.9). Naturally, temperature significantly affected the lag period ($p=0.001$), a difference of approximately 120 to 150h between W15 and W6. Overall, both samples produced similar plots, with similar fluctuations in matching sample weeks.

An additional graph in figure 1.9 was created to compare the effect of temperature on slope values. Although slopes were less visually distinct than lags between 15°C and 6°C, a one-way ANOVA clearly revealed that the incubation temperature of the plates significantly affected the slope parameters of W15 and W6 samples ($p=0.001$). February 26 (week 2) had the largest slope difference of 0.019 and March 11 (week 5), the smallest difference of 0.0095. The mean slope values per sample week also produced similar patterns of variation between temperatures. The W15 slope values exhibited greater variation between weeks than the W6 slopes.

Principal components analysis on lag and slope revealed that temperature significantly affected the arrangement of samples on three-dimensional PCA plots (FIG. 1.10, 1.11). As previously mentioned, there were relatively large differences between

W15 and W6 sample mean lag values (FIG. 1.9). But, rather than testing the temperature effect with a single mean value per sample, PCA was used to take advantage of the 95 substrates within each sample plate. With multivariate analyses, a great deal of information is incorporated into each investigation, yet it is reduced to a reasonable quantity of information, which is then more easily interpreted. For lag, PCA identified three principal components accounting for 96% of the variation between samples (FIG. 1.10). The W6 samples had very high scores along PC1 which accounted for 87% of the variance. Using lag, temperature was able to produce two distinct sample groupings (W6 versus W15) along this axis ($p<0.001$). Principal components 2 and 3 were unable to significantly discriminate between W15 and W6 ($p=0.412$) and ($p=0.812$).

The pins that represent W15 and W6 slope scores along PC1 essentially reversed their position with the lag scores from the previous PCA graph (FIG. 1.11). The W15 samples had high loadings, due to their high slope values along this axis. The slope values of W6 samples were very low and thus, had very low loadings. PC1 accounted for 67% of the variations amongst substrates, and as expected, significantly discriminated samples of different temperatures ($p=0.001$). The other two axes did not show significant separation of samples.

DISCUSSION

Biolog GN plates are not adversely affected when incubated at low temperatures. At 6°C, higher average well OD values resulted with GN plates compared to SF-N plates following the 248h reading. But, the GN plates incubated at 25°C for 48h produced a

reading similar to the final average well OD value of GN plates at 6°C. Therefore, the maximum average well OD at 6°C for GN plates was not decreased compared to 25°C. Prior to 248h of incubation, little colour development had occurred in the wells of GN plates. The average well OD due to colour and turbidity readings in GN plates prior to 248h might not have been sufficient to produce a higher average well OD than that of SF-N plates. Although the last measured average well OD was considerably lower in SF-N than with the GN plate, the use of SF-N plates as a control for testing dye chemistry at low temperatures may not be appropriate for comparison with GN plates. Although they are identical to each other, the lack of colour in SF-N plates makes it difficult to compare OD readings accurately and effectively. The best control for checking the effect of a lowered temperature on the dye chemistry of GN plates is to inoculate GN plates with a matching sample at a temperature near or at the recommended incubation temperature, as was done in this study. Thus, any CLPP differences found throughout the study between samples incubated at 15°C and 6°C using various parameters for measurement were not due to the limitations of the dye chemistry of the GN plates. The tetrazolium dye was still reduced at 6°C, and the change in colour could be measured following carbon utilization.

The low incubation temperature of Biolog plates did however affect the rate of colour development and the selection of time as to when colour development of a sample should be measured. As expected, a decrease in temperature of 9°C produced a substantial difference in the time required to reach equal stages in colour development using either AWCD or AOD parameters. Naturally, a lower incubation temperature means that a later reading must be used to get a comparable measurement.

A midpoint value of AWCD 0.5 for an entire plate measured the average colour development when a large percentage of the wells had turned positive. For W15, AWCD 0.5 was reached at 90h, and 275h (about 11.5 days) for W6. The alternative approach to reference point readings is the measurement of AOD when 25, 50, and 75% of the wells in the plate have turned positive. For all W15 samples combined, the times correlating to the three reference points were 25h, 40h, and 60h respectively. These times correlate to AWCDs of 0.02, 0.04, and 0.15 absorbance units. For W6 samples, 80, 145, and 225h represent the incubation period required to reach AOD25, 50 and 75. These translate to AWCDs of 0.02, 0.06, and 0.32 respectively. To further appreciate the use of early versus late measurements, PCA was used in an attempt to visualize how temperature affected the timing of plate readings, and the effects of the incubation period on community profiles.

Ordination plots of PCA revealed that AOD25, 50 and 75 were not able to significantly discriminate between samples incubated at different temperatures. However, in all AOD plots, the first two sampling weeks were remotely positioned from the remaining samples. These samples are obvious in the AOD25 plot, with negative scores along PC1. In AOD50 and 75 plots, both samples have negative scores along PC2 and are located towards the left of the graph. Supporting data (chapter 2) verified that AODC, SPC, total and fecal coliform, and bacterial activity counts were elevated in these samples. These findings likely contributed to the component scores in the analyses, and as a result, distanced them from other samples. The arrangement of sample weeks on PCA plots using the AOD parameter suggests that the river bacterial community remained unchanged following an early reading up to and including readings when 75%

of wells had turned positive. The AWCD 0.5 did however produce a PC plot where W15 samples were positioned to the left along PC2 with negative scores. W6 samples had positive scores along this axis, and were positioned to the right of the plot. With the formation of two major groups, this plot suggests that at AWCD 0.5, the substrate utilization pattern represents a profile due to a change in the bacterial community of the plate over time, and not an intrinsic difference in the community patterns due to incubation temperature. Smalla *et al.* (24) supports this idea. Their results show that following a 48h incubation period at 28°C, a single population of the inoculum dominated in almost all wells and this population was likely responsible for the Biolog profile. They provided supporting evidence of this theory by using temperature gradient gel electrophoresis (TGGE), and denaturing gradient gel electrophoresis (DGGE) profiles of the 16S rRNA gene amplified fragments from wells containing different microbial communities. With these techniques, shifts in the microbial community structure that occurred during incubation were monitored. They discovered that the structure of the original community changed following a prolonged incubation period, and selected members of the community overgrew the others. These were revealed as responsible for the plate profile. For the Cornwallis River community, incubation temperature was the only difference between W15 and W6 samples; therefore any profile differences picked up using AWCD 0.5 were likely due to enrichment that took place in the wells. This measurement is likely too late to detect true metabolic diversity of the community before sample enrichment has occurred. Analyses emphasized that an AWCD greater than 0.1-0.2 at 15°C, and 0.3 at 6°C, generally picks up substrate utilization by a portion of the community that has already been enriched in those wells. These values may appear very

low, but the AWCD is calculated using the absorbance readings for the entire plate. So even if 50% of the wells are positive, the absorbance readings for the remaining wells could be near zero. Therefore, a parameter that represents an early measurement, prior to sample enrichment, is better for the study of community diversity at any temperature.

The separation of substrates into groups with low, middle, and high temperature characteristic values revealed that the rate of colour development in particular wells was more sensitive at 6°C than at 15°C. There was significant separation between each group ($p=0.001$). The group of substrates with the highest temperature characteristic value had the largest decrease in rate of colour development due to a low incubation temperature. The group did not constitute a single substrate type. It consisted of 11 substrates belonging to 5 different types: 5 carbohydrates, 3 carboxylic acids, an amide, amino acid, and an aromatic chemical. With this combination, no conclusions could be made about the correlation between a decreased rate of colour development and substrate type due to a shift in temperature. The substrates classified in middle and low temperature characteristic groups were also not comprised of a single substrate guild. Thus, no conclusions were reached concerning a particular group of substrates and a range of temperature characteristic values for those groups. There were 90 substrates categorized in one of the three groups, leaving 5 substrates unclassified. These substrates were not utilized at all by the organisms present in those wells (Table 2). Since the Biolog assay was designed to identify pure cultures and a number of substrates present on the GN plate are not always readily available in a natural environment, it is not surprising that a small number of substrates out of 95 were not be used by any of the members in the community. Ultimately, most substrates were utilized at both temperatures, but at 6°C,

some substrates displayed a larger decrease in the rate of utilization than others. Thus, caution must be exercised in temperature studies with the Biolog assay. Specific substrates react differently to decreases in temperature, which adds a layer of complexity to the use of CLPPs. Such complications distracts from the indigenous community that the researcher is attempting to work out.

The lag phase likely represents a period of time when the community is adjusting to a particular substrate by inducing enzymes required to metabolize it. Lag is therefore an early response measurement, which may better reveal community diversity if enrichment has not already taken place. Limitations are accompanied by early measurement. Wells with no colour development produced infinite lag values. Thus, 33 substrates were excluded from any analysis related to lag phase and they were coded as missing values for multivariate analyses. Lawley and Bell (19) experienced similar difficulties in their study because all except 14 wells produced infinite lags. Despite the low number of substrates, which could affect the interpretation. PCA revealed a chlorine effect on lag. A decrease in inoculum density and the number of colony types increased the lag period until this population recovered after 36h. The effect of temperature on lag was as expected in this study. The lag phase was clearly greater for samples incubated at 6°C than for those incubated at 15°C. W6 samples produced high lag values and therefore loaded high on PC1. As suggested earlier, lag represents sample adjustment. The increased period of time may have been required for the community to adjust to a change in temperature, altered substrate concentration and availability, and in general, to a complete change from its natural environment.

Slope is another kinetic measurement which takes into account early as well as late readings. Working with slope is much more labour intensive than the one-time, or reference point readings, but eliminates the uncertainty of the optimum times to take OD readings. As expected, slope decreased with a decrease in incubation temperature. The rate of colour development did slow down in cooler temperatures, and this was clearly illustrated using PCA. The W15 samples with steeper slopes produced much higher scores than W6 samples. Once again, W15 samples are shown with very high positive scores along PC 1 (FIG. 11). Glimm *et al.* (8) stated that extreme values are usually responsible for most of the variation and are reflected in the first PC. PCA loads up PC1 with the variable where the magnitude is greatest. In this study PCA reveals the obvious, the higher the temperature the greater the slope. The use of lag with temperature presents a similar challenge. PC1 reflects the intensity of a response and not the pattern resulting from substrate use. A decrease in temperature resulted in longer lags hence higher scores along PC1. Apart from picking up large differences between lags at 6°C and 15°C, it provided little information about the river community. In this study, the investigation of temperature effects highlights the limitations and shortcomings associated with slope and lag.

CLPPs can be performed at various temperatures as low as 6°C, but extreme caution must be used in their interpretation. Whether choosing to use AWCD or AOD reference points, parameters that measure early colour development appear better than those that use later readings. Complications arising from the enrichment process may be prevented, and an earlier reading (such as lag or AOD25) presents a better representation of the indigenous bacterial community. Additional information was gathered on the

thermodynamic effects of substrates using Arrhenius plots. By categorizing substrates into groups according to their temperature characteristic value, Arrhenius plots of their colour responses revealed that decreases in temperature results in a range of substrate reactions by the sample community. This adds a layer of complication to the interpretation of CLPPs, especially when temperature is involved. PCA may not be the best choice for analysis in this case, as PCA focuses on the magnitude of the differences between lags and slopes of each incubation temperature and overlooks community similarities. Kinetic parameters, lag and slope, do not appear to provide any advantages when dealing with different temperatures.

Table 1. List of substrates classified in "high" group of temperature characteristic values.

Substrate	Well	Substrate type	Average Temperature characterisitic
N-acetyl-D-glucosamine	A8	carbohydrate	28249
erythriol	B1	carbohydrate	46580
alpha-D-lactose	B8	carbohydrate	58414
L-rhamnose	C5	carbohydrate	53272
turanose	C9	carbohydrate	38963
acetic acid	D1	carboxylic acid	40169
propionic acid	E8	carboxylic acid	37022
sebacic acid	E11	carboxylic acid	35826
alaninamide	F4	Amide	71180
glycyl-L-aspartic acid	F11	amino acid	28084
thymidine	H4	aromatic chemical	83818

Table 2. List of substrates with a temperature characteristic value of zero and not included in any group.

Substrate	Well	Guild
formic acid	D4	carboxylic acid
alpha-hydroxybutyric acid	D10	carboxylic acid
alpha-keto valeric acid	E3	carboxylic acid
malonic acid	E5	carboxylic acid
L-phenylalanine	G5	amino acid

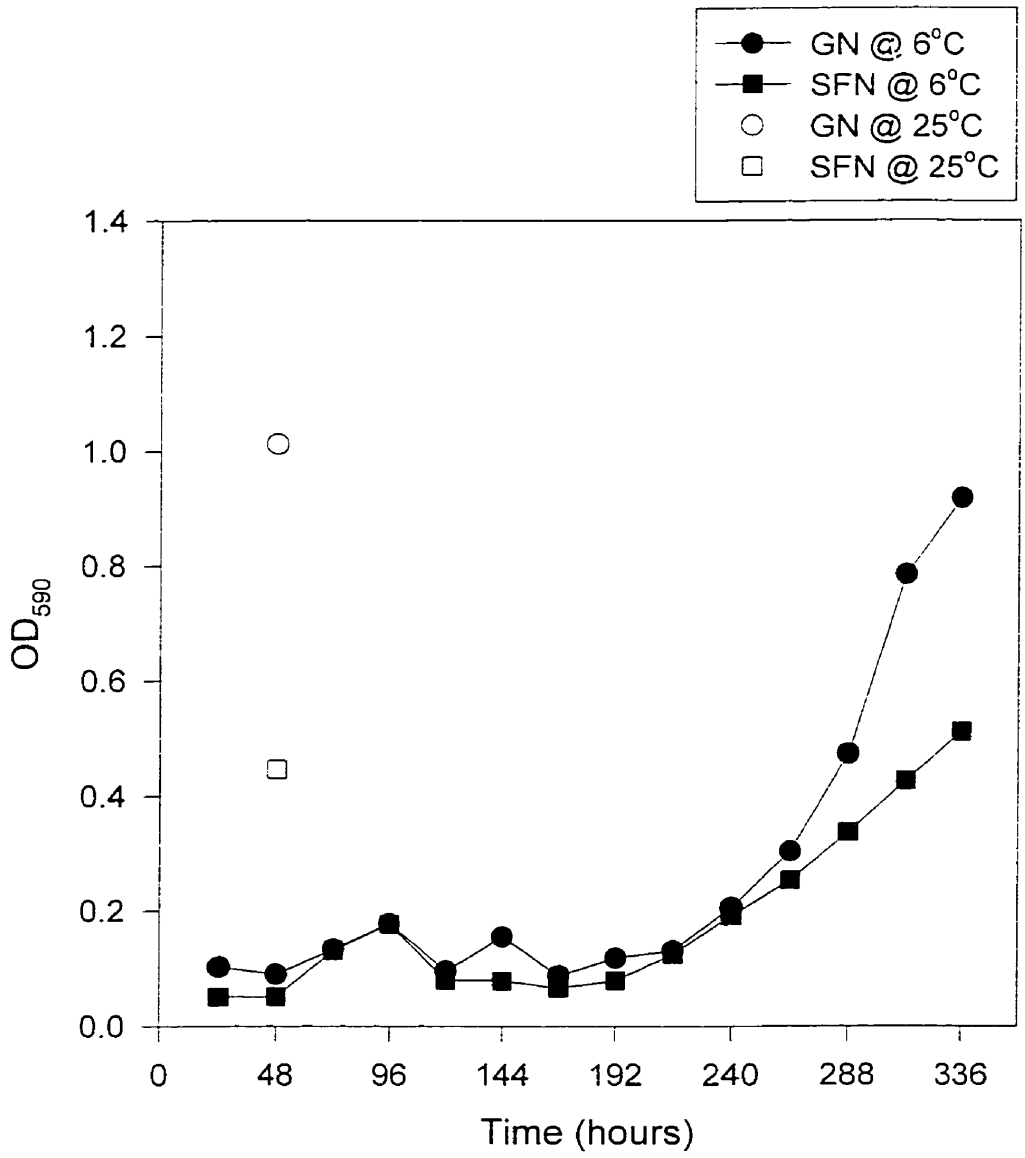

FIG. 1.1. Optical density versus time curves for GN and S-FN plates inoculated with identical samples and incubated at 6°C. The hollow symbols were incubated at 25°C.

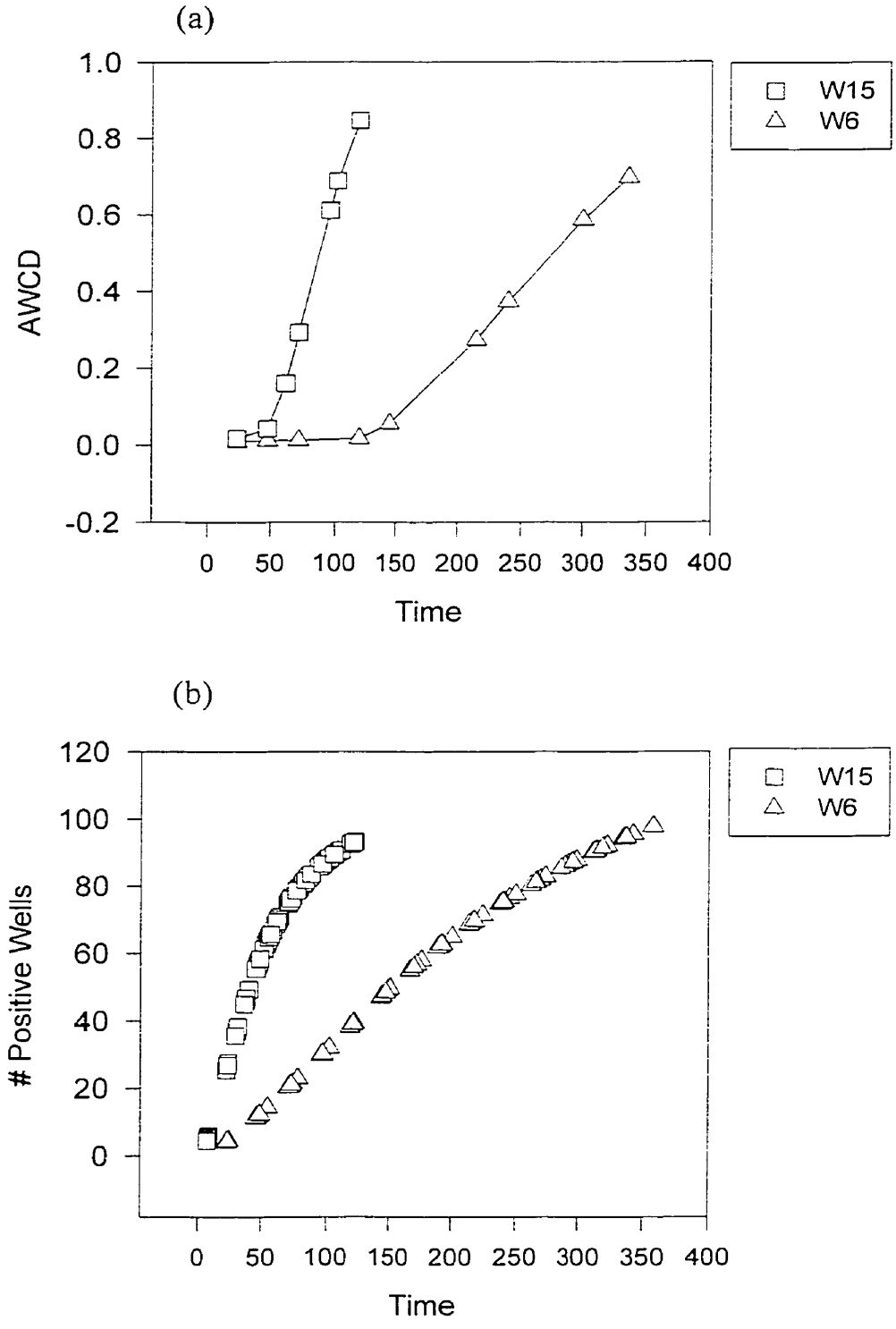

FIG. 1.2. The (a) AWCD versus time curves and (b) # positive wells versus time curves for W15 and W6 samples.

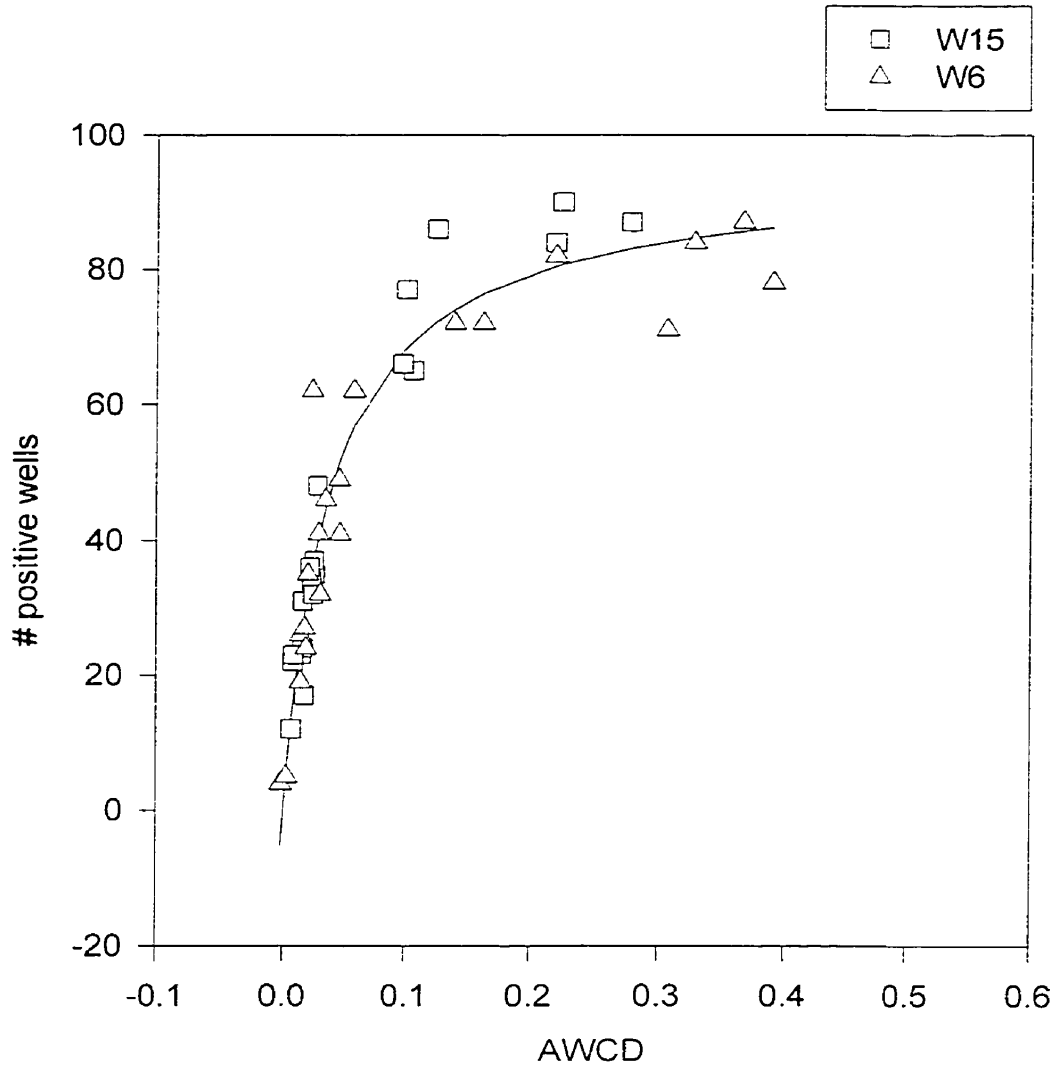

FIG. 1.3. Curve illustrating # positive wells versus AWCD values for W15 and W6 samples.

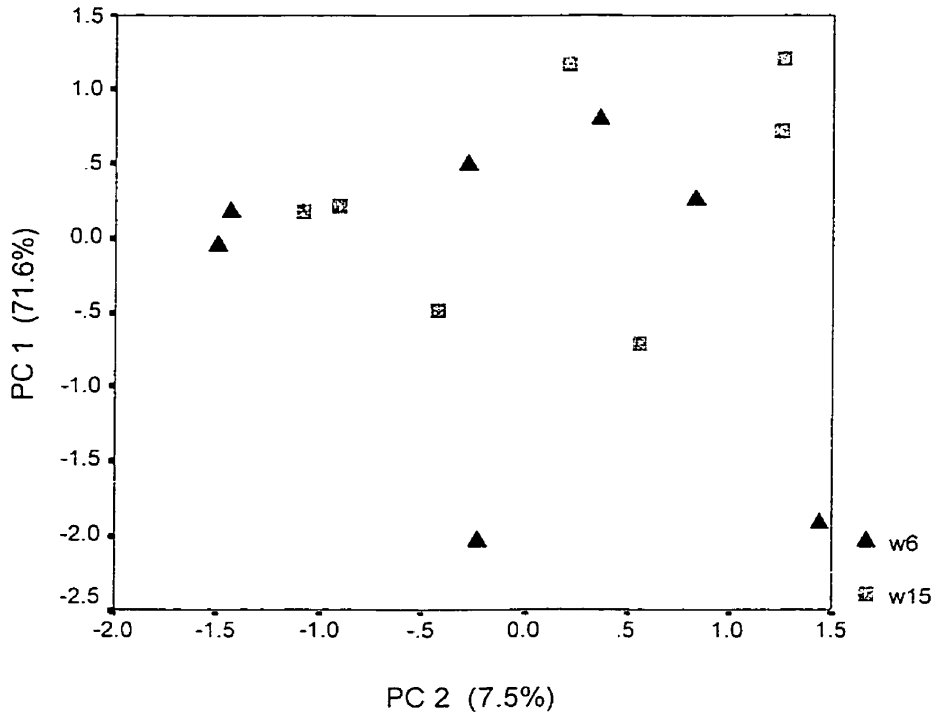

FIG. 1.4. Ordination plot of PCA using AOD25 values for W15 and W6 samples.

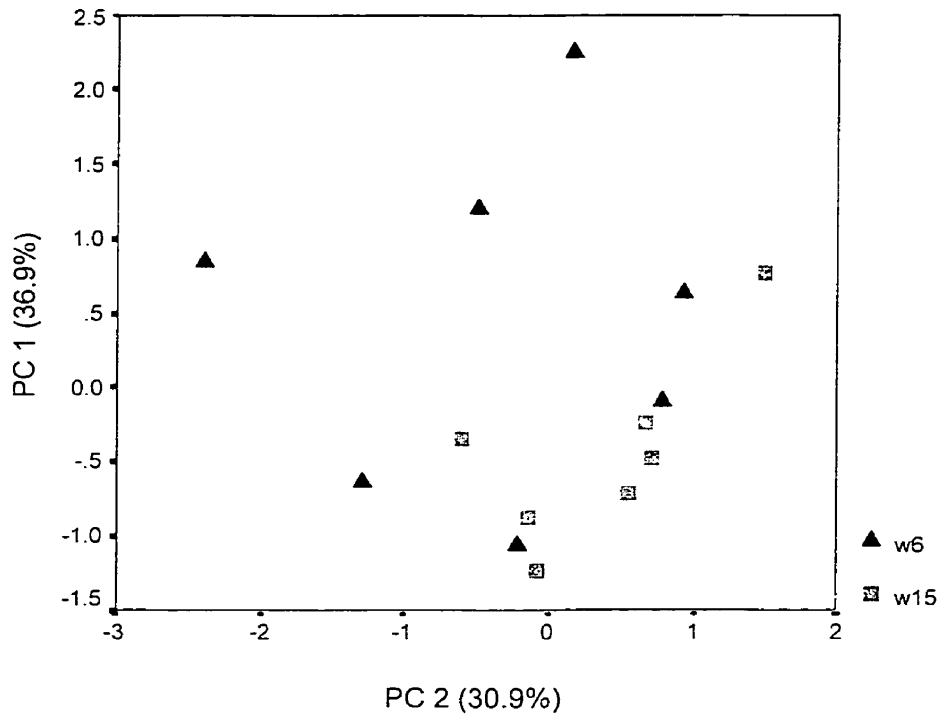

FIG. 1.5. Ordination plot of PCA using AOD50 values for W15 and W6 samples.

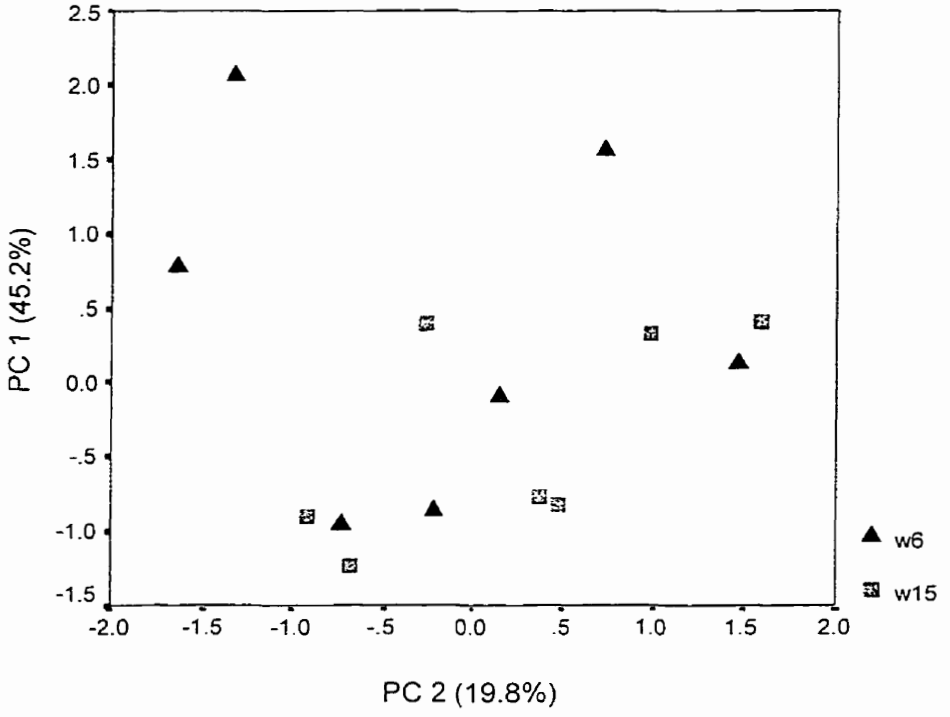

FIG. 1.6. Ordination plot of PCA using AOD75 values for W15 and W6 samples.

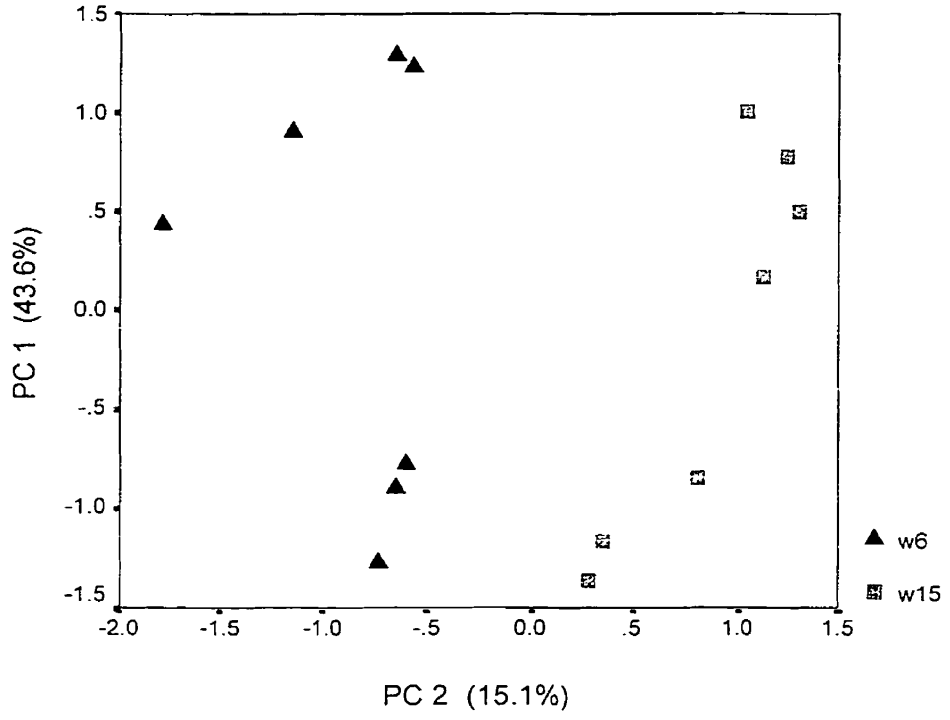

FIG. 1.7. Ordination plot of PCA using AWCD 0.5 values for W15 and W6 samples.

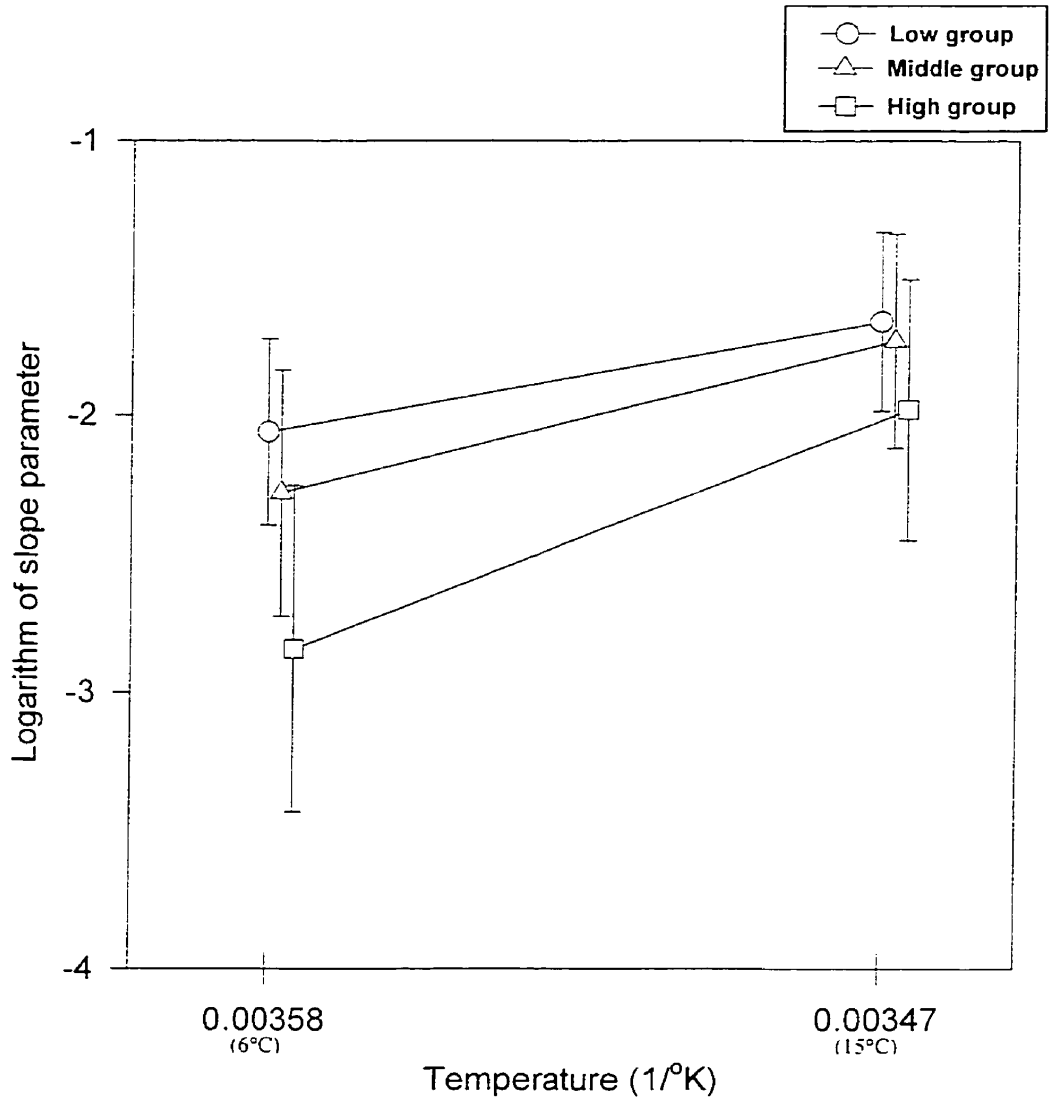

FIG. 1.8. Arrhenius plot using the logarithm of slope values to plot the groups of substrates with low, middle and high temperature characteristic values. Temperature is denoted in 1/°K.

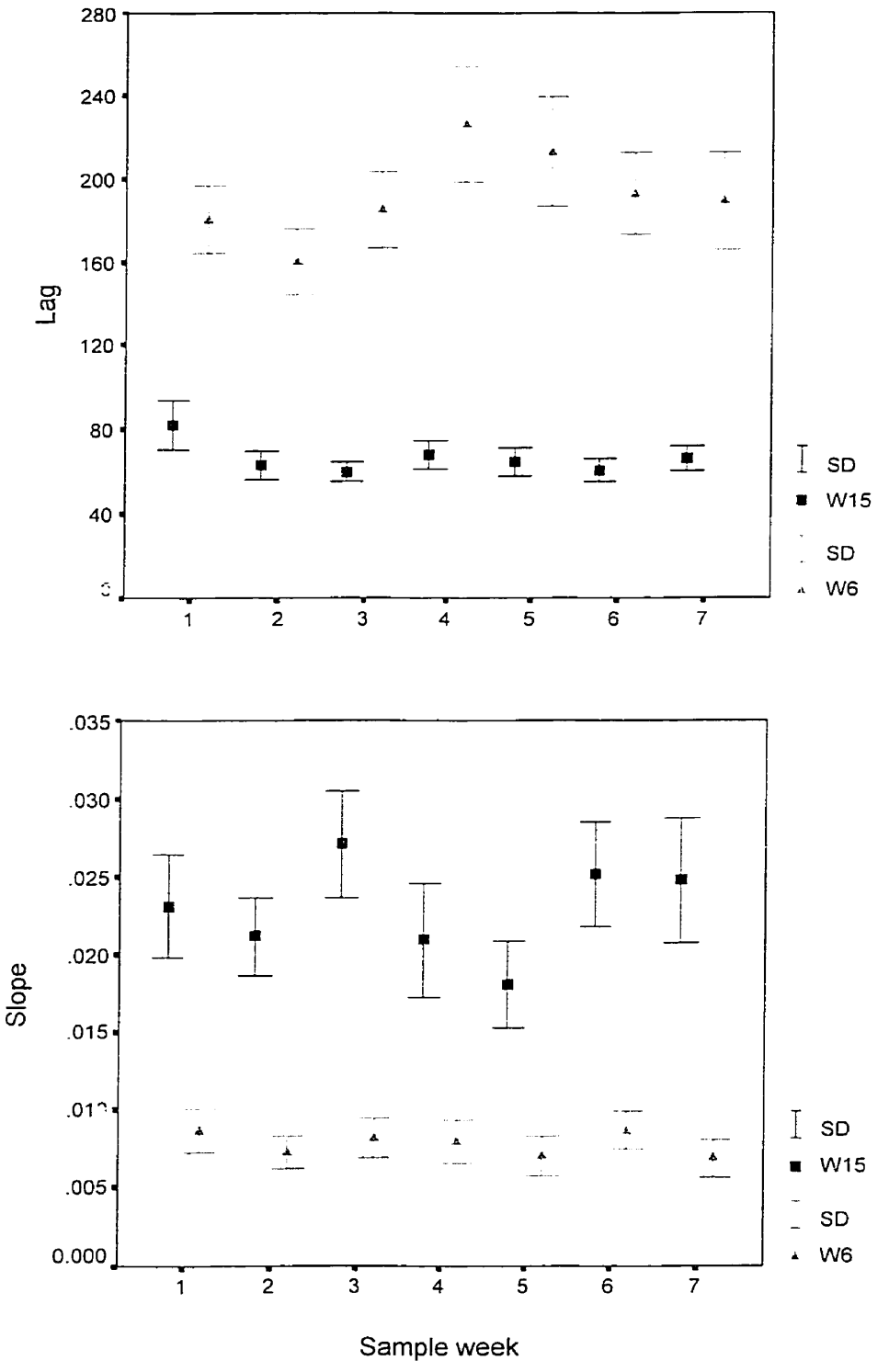

FIG. 1.9. A comparison of mean lag and slope values per week for W15 and W6.

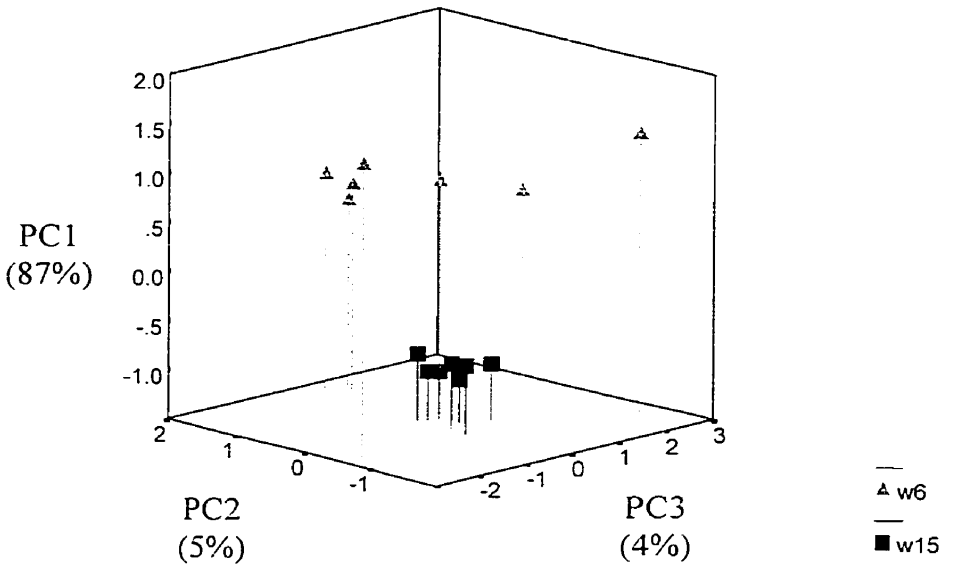

FIG. 1.10. Ordination plot of PCA using lag values from W15 and W6 curves.

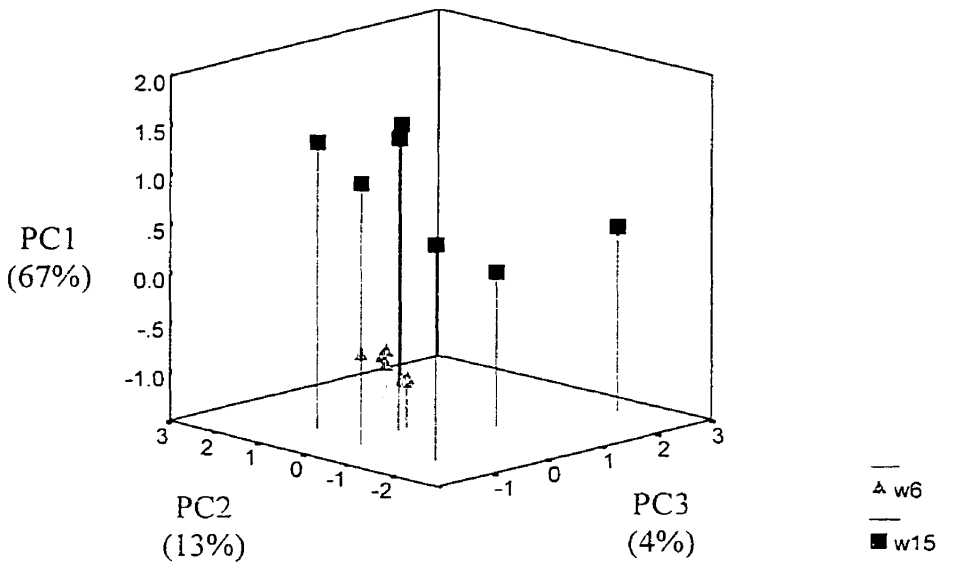

FIG. 1.11. Ordination plot of PCA using slope values from W15 and W6 curves.

Chapter 2: The bacterial community of the Cornwallis River

INTRODUCTION

The Cornwallis River is 40 km long and empties into the Minas Basin of the Bay of Fundy. It is one of the main sources of water for irrigation of farmland located along this river, but unfortunately, the Cornwallis River also receives some municipal, agricultural, and food processed wastes as it empties into the estuary. Sewage treatment facilities, fruit and vegetable canneries, a meat packing plant, and poultry processing plants are some of the contributing sources. Studies by Friends of the Cornwallis River (unpublished data), with the assistance of the Centre for Estuarine Research at Acadia University revealed unfavourable results. Specifically, low dissolved oxygen levels were found, which could create harmful habitat for trout and salmon. Elevated biological oxygen demand levels were also reported, implying a greater input of organic materials than the microorganisms in the river were able to utilize. During the summer months, extremely low water levels due to hot, dry weather and heavy use within the river's main watershed were also a concern for those dependent on the Cornwallis River.

Community analyses using Biolog

Biolog community level physiological profiles have been used in several studies to measure spatial (16), temporal (13, 16) or habitat community differences (9, 33). The contribution of populations to the Biolog pattern has also been tested (24, 28) using genetic fingerprinting techniques. Various studies have been performed to determine which parameters best illustrate these differences. Garland and Mills (7) introduced the

application of several single point OD readings by taking the average well colour development (AWCD) of the Biolog plate. Many have followed this approach (8, 16, 17) or opted for the reference point AWCDs (5). Kinetic profiles obtained from positive responses over time (11), or the kinetic parameters extracted from the substrate response curves (10, 19, 28), have also been used extensively to avoid having to select a time to read the plate OD. In this chapter, both AWCD and kinetic parameters are applied to CLPPs of summer and winter Cornwallis River water samples.

Objectives

This chapter examined several parameters as a means of measuring differences between summer and winter bacterial communities of the Cornwallis River. Various AOD reference time points, AWCD and kinetic parameters, slope and lag were used to measure this shift in the river community. In an attempt to provide additional information about the river, acridine orange direct counts (AODC), thymidine incorporation and coliform counts were performed on both summer and winter water samples. The Biolog identification profiles of randomly selected colonies were subjected to cluster analysis and a structural/taxonomic tree created. This would reveal major differences in the culturable members of each community. The final goal was to use the results from this manuscript to facilitate the development of new methods in the study of bacterial communities.

MATERIALS AND METHODS

Sample Collection

The sample site chosen for this study was the Cornwallis River (45° 05'N, 63° 35'W). Sample collection was identical to the method used in chapter 1. From July 10 to August 28, 1995, seven samples were collected from the river. Between February 13 and March 25, 1996, an additional seven samples were obtained. The temperature and depth of the river were recorded on site. All samples were immediately carried back to the laboratory and processed within 2h.

Biolog Multisubstrate Plate Inoculation

Whole river samples were diluted (1/5) with phosphate buffered saline (PBS) and inoculated into Biolog GN microtitre plates (BIOLOG Inc., Hayward, CA). Two plates were inoculated for each sample week. Both summer and winter samples were incubated at 15°C (S15 and W15). The optical density of each well was measured every 6-8h for 120h. The optical density was measured using the Series 750 Microplate Reader (Cambridge Technology, Inc.), in combination with the Microlog 3N 3.01A program Version DE (BIOLOG Inc., Hayward, CA).

River Supporting Data

Additional tests on summer and winter samples of the Cornwallis River were performed to provide background information about the river water. These tests were as follows: (i) tritiated thimidine incorporation, (ii) direct microscope counts, (iii) coliform

counts and, (iv) plate counts. The calculations for these four tests are found in Appendix V.

Tritiated thymidine uptake was conducted according to the instructions of Fuhrrman and Azam (3). The viable and non-viable bacterial cells were enumerated using the acridine o.ange direct counting (AODC) technique (15). The total and fecal coliforms were determined using standard procedures (Standard Methods for the Examination of Water and Wastewater, 17^{ed}, 1989) with mEndo and mFC plates. Typical coliform colonies (red with metallic surface sheen) were counted under a white fluorescent light with the plates at a 30° angle, and only those filters with 20 - 80 coliforms were used in the calculations. The total coliform counts per ml were determined by dividing the number of coliforms per sample by its volume. The fecal coliform counts were also determined using a membrane filter technique. The blue colonies were again counted under a fluorescent light at a 30° angle, and the number of fecal coliforms per sample was calculated in the same manner as the total coliform counts. Plate counts were performed to determine the number of viable/culturable bacteria in each sample. Two 1/5 TSA plates were inoculated at 20°C with each sample using a Spiral Plater, Model C (Spiral Systems, Inc., Baltimore Maryland). The bacterial density was determined by counting the colonies over a special grid that relates volume to area. It was necessary to count at least 20 colonies. The volume of the section of the grid in which the 20th colony was observed was the number used in calculating the plate counts. To balance irregularities in sample dispersal, the same segments in the opposite sector were counted. Both numbers were added and divided by the section volume constant to give the number of colony-forming units per ml of sample.

Identification profile tree

Summer and winter samples were transferred to 1/5 TSA plates using a micropipette, and evenly spread out with a glass hockey stick. Following a 24-48 hour incubation period at 20°C, 30 colonies were randomly picked using a numbered grid attached to the bottom surface of the plates, and then transferred to fresh plates. A calculator program was used to ensure randomness. Colonies from each sample were grown at the temperature at which they were isolated. Once pure cultures were obtained, these were Gram stained and identified using the Biolog system. These were then tested for their ability to grow at 5°C and 30°C. A dendrogram was created using cluster analysis, which would allow the similarity between isolated colonies to be more easily visualized. Colonies with similar identification patterns are positioned closer to each other on the tree than colonies with very different Biolog identification patterns.

Biolog data manipulations

The Biolog data obtained from W15 samples were compiled and processed using the same methods described in Chapter 1. Data obtained from S15 samples were subjected to the same manipulations as W15 samples with the exclusion of the calculation of temperature characteristic values for each substrate. All Sigma Plot transforms, the Biolog background colour correction file, sample graphs for AOD reference times, and the logistic curve parameters found in Appendices I, II, III and IV, were used in data analyses.

RESULTS

Summer versus winter using AOD, AWCD, and kinetic parameters

The AOD versus time plots were created to calculate the times required to reach the reference point readings of AOD25, 50 and 75. The S15 samples reached AOD25 following a 25h incubation period, while W15 took 20 h. Both samples reached AOD50 within 40h. An AOD of 75% positive wells was reached after 55h for S15 samples and 60h for W15 samples. The W15 had a greater rate of colour development than S15 during the first 40h of incubation. But following this incubation, the S15 samples quickly caught up and OD readings increased more rapidly than W15 samples.

A PCA on AOD25, an early measurement, revealed that S15 and W15 samples were not separated into distinct groups on either principal component ($p>0.05$). The third sample week from S15 samples was positioned at the lower right hand corner of the plot with a negative PC1 score (FIG. 2.1). With a thimidine uptake value of 1.4×10^3 cells/ml/hr on week 3, bacterial activity was decreased by about ten fold when compared to most of the remaining S15 weeks. Sampling season did have a significant effect on AOD50 on PC1 ($p=0.036$). The first three weeks of summer samples had high PC1 scores and are positioned from left to right on the top portion of the plot (FIG. 2.2). The W15 samples all produced scores between 0 and −1 on PC1, and formed a tight cluster towards the bottom of the plot. W15 samples also formed a cluster on PC2, with scores of 0 to −1, but the ANOVA test showed that the S15 and W15 scores were not significantly different ($p=0.565$). Another PCA was performed on the next reference point reading, AOD75. An ordination plot (FIG. 2.3) showed that week 1 from S15 samples, high on the first component was also found to have high total coliform levels.

All samples scored similarly on PC1 with no significant grouping of samples on this axis ($p>0.05$). W15 samples were all positioned to the bottom left corner of the plot, nearly all with negative PC2 scores. This component revealed that AOD75 detected a seasonal effect ($p=0.044$). With PC2 only accounting for 8.5% of the variance between samples, the significance of this effect is subtle. The AWCD 0.5 PCA plot showed no separation of samples on PC1, but produced two distinct groups on PC2, W15 had negative scores and S15 had positive scores (FIG.2.4). Once again, week 1 from S15 was positioned at the top of the plot, but to the right this time. The AWCD 0.5 patterns of each set of samples were significantly affected by the sampling season ($p=0.001$) on PC2, accounting for only 12.9% of the variance in the component scores.

The kinetic parameters slope and lag did show some significant seasonal differences, verified using ANOVA tests on PCA scores. Slope PC1 scores were fairly uniform among all samples except week 1 of S15, which had a high value on this axis. An ANOVA confirmed no seasonal effects on PC1 ($p=0.156$). Slope was able to significantly group samples according to season on PC2 ($p=0.001$), with S15 towards the left and W15 on the right of the plot (FIG. 2.5). Another PCA plot (FIG. 2.6) revealed a seasonal effect with lag, barely on PC1 ($p=0.047$) but clearly on PC2 ($p=0.001$). PC2 accounts for a mere 8.5% of the variance, therefore the seasonal effect is present but slight.

Following the examination of these analyses, the question of whether there was a shift in the community between seasons was reconsidered, given that among the reference point readings, only AOD50 picked up seasonal differences on PC1 with W15 grouped in a tight cluster and S15 samples dispersed randomly along this axis. Both

AOD75 and AWCD 0.5 had significant seasonal effect on PC2, but this component accounts for less variance in the analysis. Slope and lag were able to pick up subtle community kinetic differences on PC2, and lag was affected by season on PC1.

Supporting data

Several graphs were created to compare S15 and W15 data, and to highlight any differences between samples. The AODC and plate count graphs (FIG. 2.7) clearly show that S15 had higher counts when using the AODC method for counting, but plate counts were consistently higher for W15 than in S15 samples. The tritiated thymidine uptake varied between samples (FIG. 2.7). The S15 week 4 sample was almost ten times higher than the second highest uptake value recorded in the first week. It was noted that two days prior to sampling, heavy rain flooded the area and a new sample spot was chosen for week 4. The total coliform counts (FIG. 2.8) were frequently more elevated in W15 samples, with weeks 1 and 2 showing a substantial increase. There were no major trends to highlight for fecal coliform counts except that W15 counts were on average just as high as S15 counts.

Biolog identification profiles and cluster analysis

From the thirty random picked colonies from the summer sample, one isolate would not produce any colour development in the Biolog plates and as a result was removed from the list. The same occurred to two of the winter isolates. Cluster analysis was performed to assess the relative similarity between the isolates. The Pearson correlation coefficient was used as a similarity index and the between-groups average

linkage method to cluster the cases. The results of the cluster analysis are shown in a dendrogram (FIG. 2.9). The isolates connected by branches near zero are more closely related than those whose branches are located to the right of the tree. The dendrogram rescales the actual distances to numbers between 0 and 25, preserving the ratio of the distances between steps. All isolated colonies were able to grow at 30°C, and only those marked with an asterisk had the ability to grow at 5°C as well. These isolates consisted of 16 from W15 and 15 from S15. The top two clusters (1 and 2) are made up of colonies isolated from both seasons but unable to grow at 5°C. The next group of isolates, indicated as cluster 3, consisted of colonies exclusive to W15. All except one isolate were unable to grow at 5°C. The bottom two clusters, 4 and 5, contained isolates from both seasons that were very closely related to each other. These isolates were identified using Biolog and were all members of the following species: *Pseudomonas vesicularis, Pseudomonas diminuta, Pseudomonas marginalis,* and *Weeksella zoohelcum.*

DISCUSSION

The AOD and AWCD parameters revealed unexpected results about the river bacterial community. A shift in the community between summer and winter months was predicted, but the results did not support this theory. As previously mentioned, AOD25 is an early reading; therefore it measures intrinsic differences between samples. The PCA on this parameter revealed that AOD25 was unable to group the samples by season on either PC. Therefore, the original inoculum in S15 is very similar to that of W15 and the river communities do not change over time. A slightly later measurement with AOD50 did show a seasonal effect. The significance picked up on PC1 ($p=0.036$) is

believed to be due to the high component scores of weeks 1, 2 and 3. These were positioned at the top of the PCA plot and likely contributed to most of the variance reflected in PC1. The supporting data from these two sample weeks did not reveal any unique results. The reason for their very high component scores is unknown. W15 samples group together on PC2 using AOD75. ANOVA confirmed that this grouping of W15 was in fact significant ($p=0.044$). The elevated fecal coliform count on week 1 may explain the high PC1 scores and separation of this sample from the others. Although the bacterial numbers in the inoculum were not elevated, high coliform counts could have been enough to create a very different community profile in the plate. The AWCD 0.5 significantly grouped S15 and W15 samples separately on PC2. This reference point reading may have been picking up early enrichment in the wells. AWCD 0.5 translates to 65 - 75h of incubation, when more than 80% of the wells had gone positive. This parameter may be revealing slight community differences that have been enhanced through enrichment.

Slope and lag both revealed subtle differences in the rate of colour development between S15 and W15. S15 had a shorter lag period and a slightly higher rate of colour development than W15. Lag is considered an early response measurement, and it reflects the phase during which summer and winter samples become activated or adjust to the substrates in the plate. The W15 samples were obtained from a river near freezing temperatures and then incubated at 15°C. Therefore, W15 required a longer incubation period to adjust, but quickly showed signs of substrate utilization similar to S15. For this reason PCA illustrated significant kinetic differences between S15 and W15 samples.

Nedwell (20) supports suggestions that at low temperatures, growth rate under low nutrient conditions is more dependent on substrate concentration than at higher temperatures. Thus if the availability of substrates is decreased, growth of bacteria will be greatly reduced. It is possible that the W15 community was responding to such a change when inoculated in Biolog plates but soon recovered, and this response was quantified by the slope and lag parameters. Therefore, although slope and lag detected differences in CLPPs, these are probably not seasonal community shifts, but differences in how the river community adjusted to the Biolog substrates.

Figures 2.7 and 2.8 both show that culturable members of the winter community occasionally produce higher cell counts than those of the summer community. The direct counts were consistently higher for S15 than W15, but for the remaining tests, both summer and winter samples showed comparable results. Except for one exceptionally high thimidine uptake value reported in S15, bacterial activity counts show that the river community is not restricted by a lowered temperature and successfully adapts to the changing climate.

Based on the identification profiles of selected bacterial colonies, a shift in the bacterial community from summer to winter does not appear to occur. Isolates clustered based on their substrate utilization pattern, and many clusters consisted of a mixture of S15 and W15 isolates. A single cluster was made up of only W15 samples. The remaining obvious clusters contained both summer and winter isolates. The dendrogram shows that the culturable bacteria in the river do not change between the summer and winter months. These findings also support conclusions found using Biolog CLPP parameters with whole river samples. The communities present in the Cornwallis River

are well adapted for growth or survival to the varying temperatures imposed on them and the river bacterial community is not significantly altered with changes in temperature.

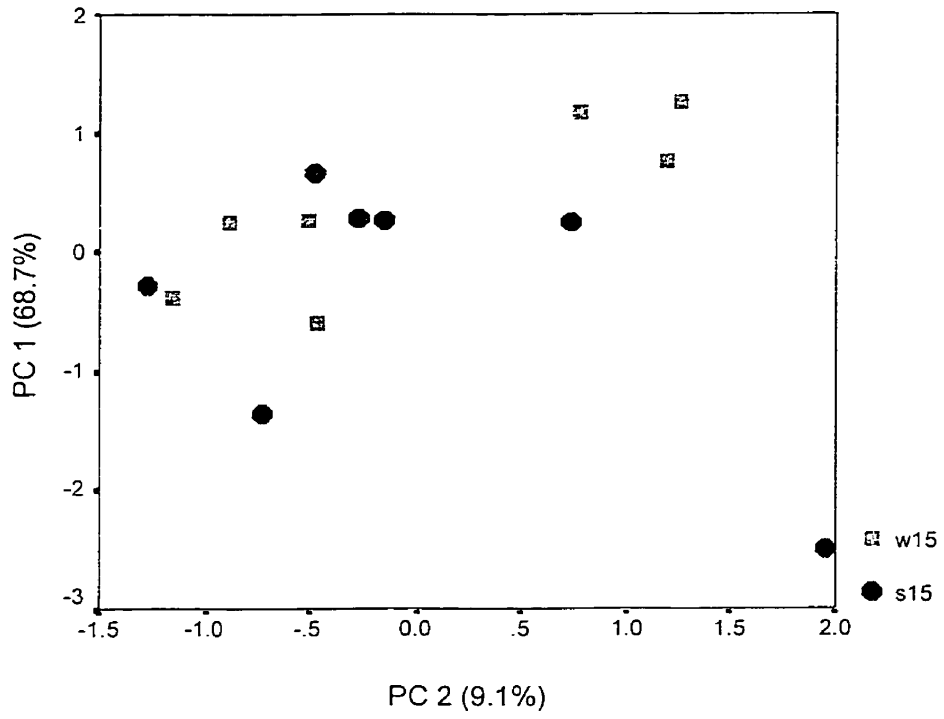

FIG. 2.1. Ordination plot of PCA using AOD25 values from S15 and W15 samples.

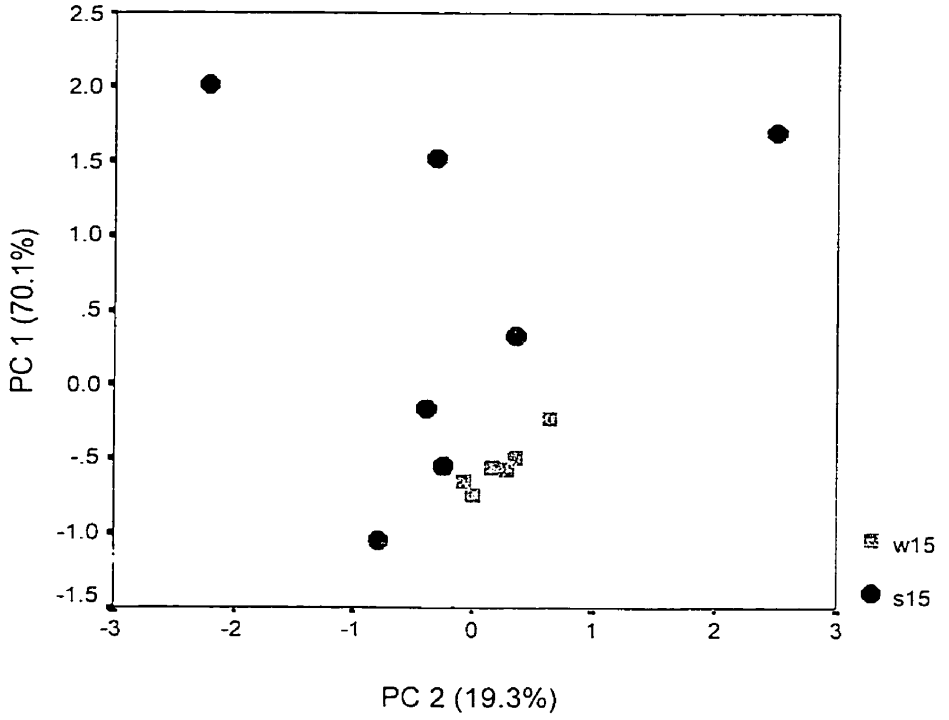

FIG. 2.2. Ordination plot of PCA using AOD50 values from S15 and W15 samples.

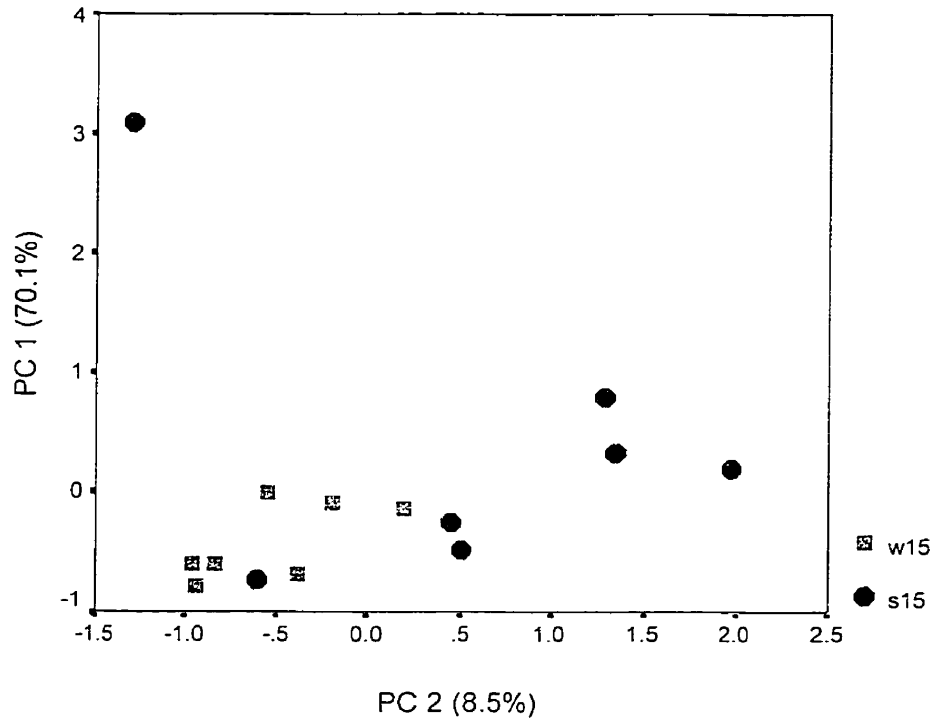

FIG. 2.3. Ordination plot of PCA using AOD75 values from S15 and W15 samples.

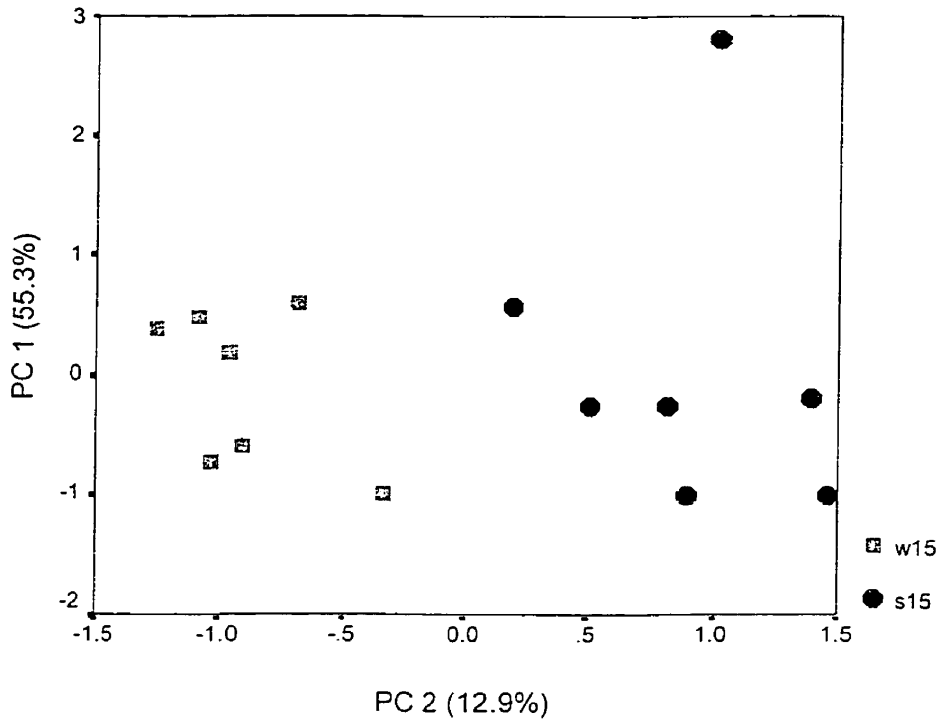

FIG. 2.4. Ordination plot of PCA using AWCD 0.5 values from S15 and W15 samples.

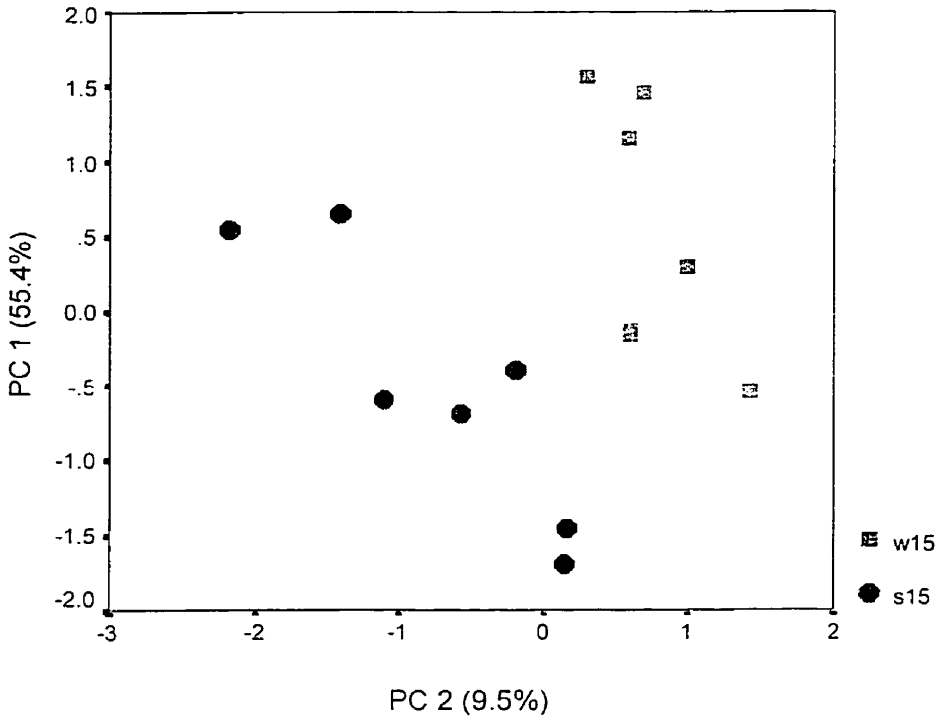

FIG. 2.5. Ordination plot of PCA using lag values from S15 and W15 samples.

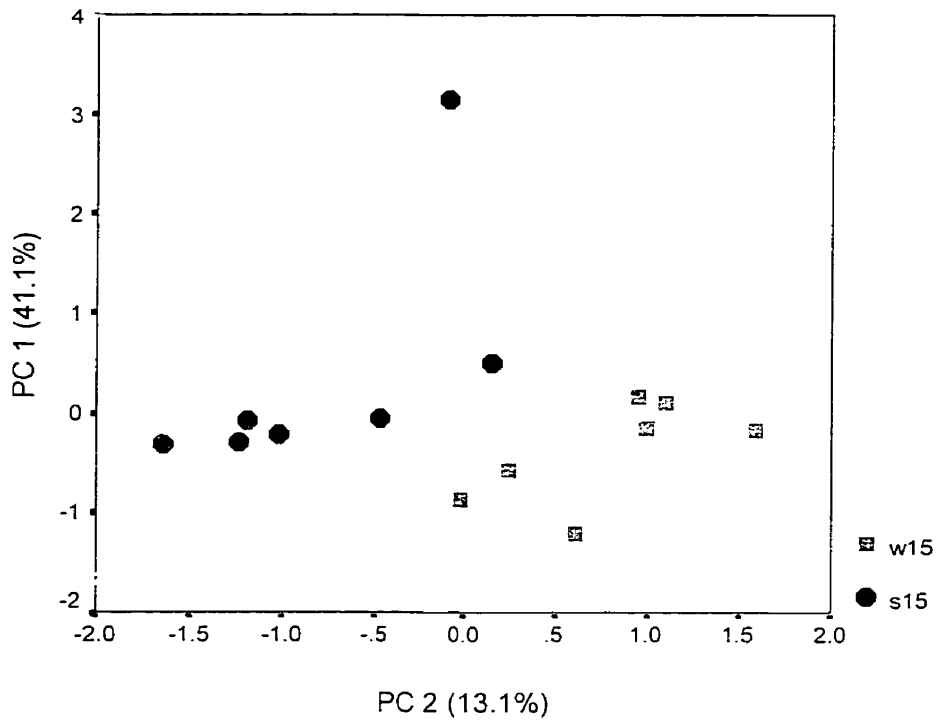

FIG. 2.6. Ordination plot of PCA using slope values from S15 and W15 samples.

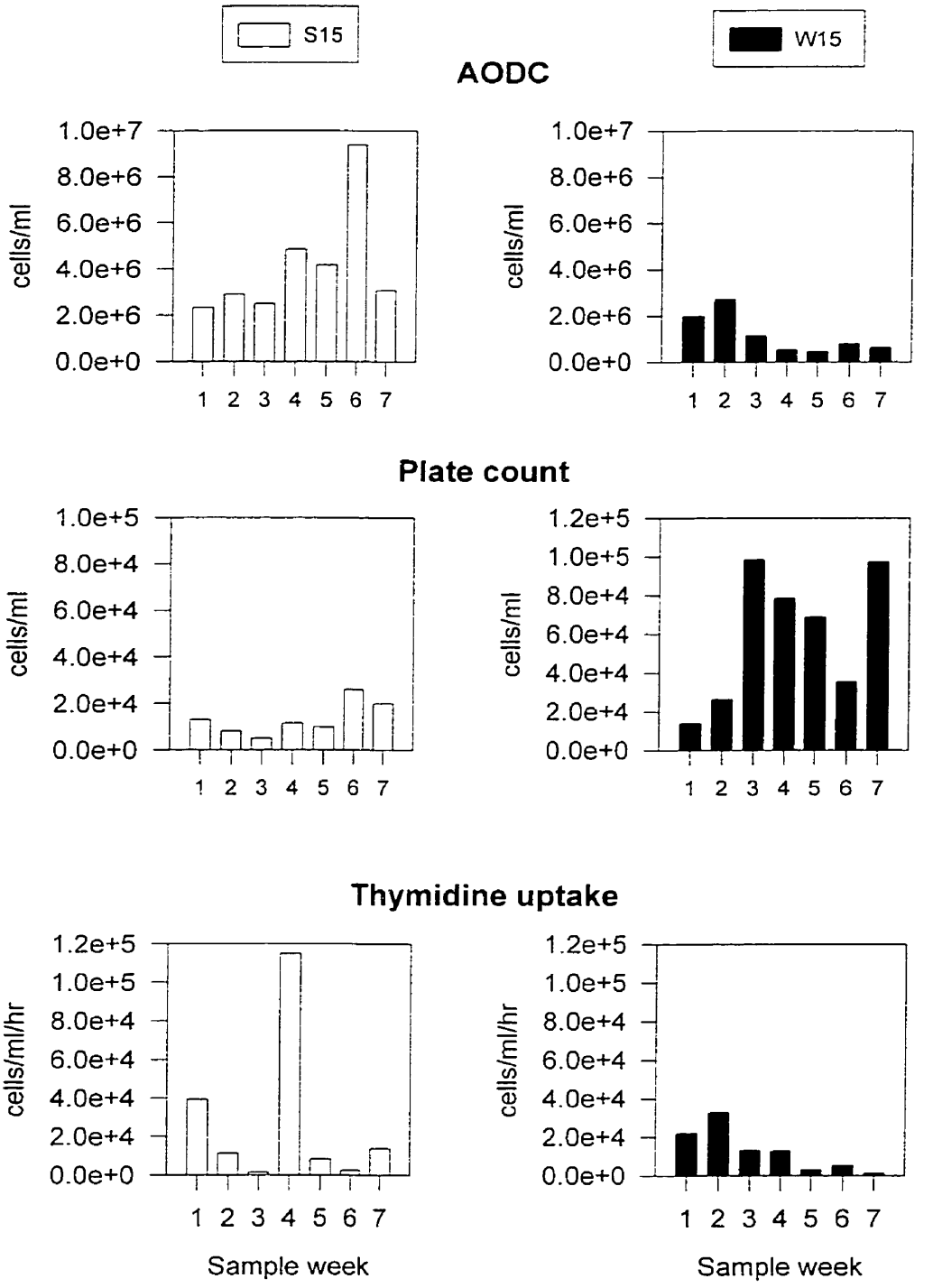

FIG. 2.7. Plots showing acridine orange direct counts, plate counts, and tritiated thimidine uptake for S15 and W15 samples.

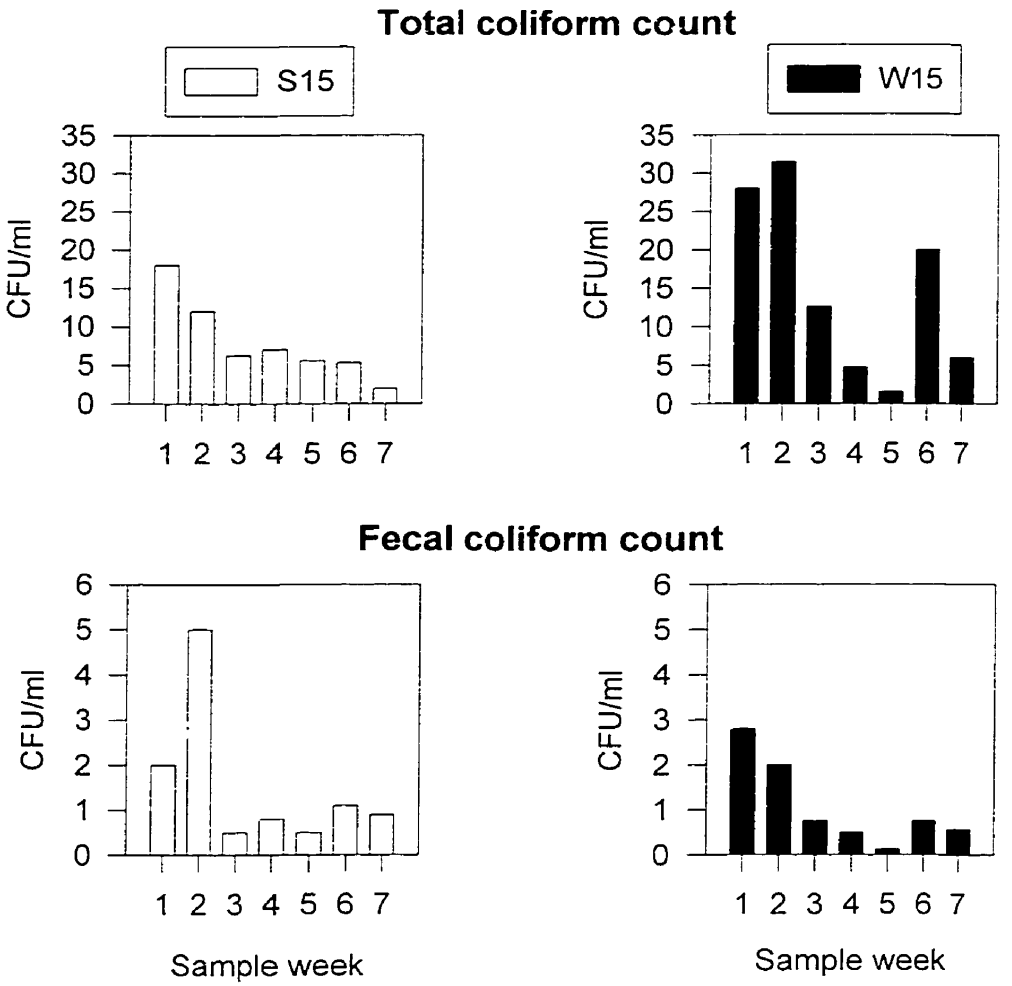

FIG. 2.8. Plots showing total and fecal coliform counts for S15 and W15 samples.

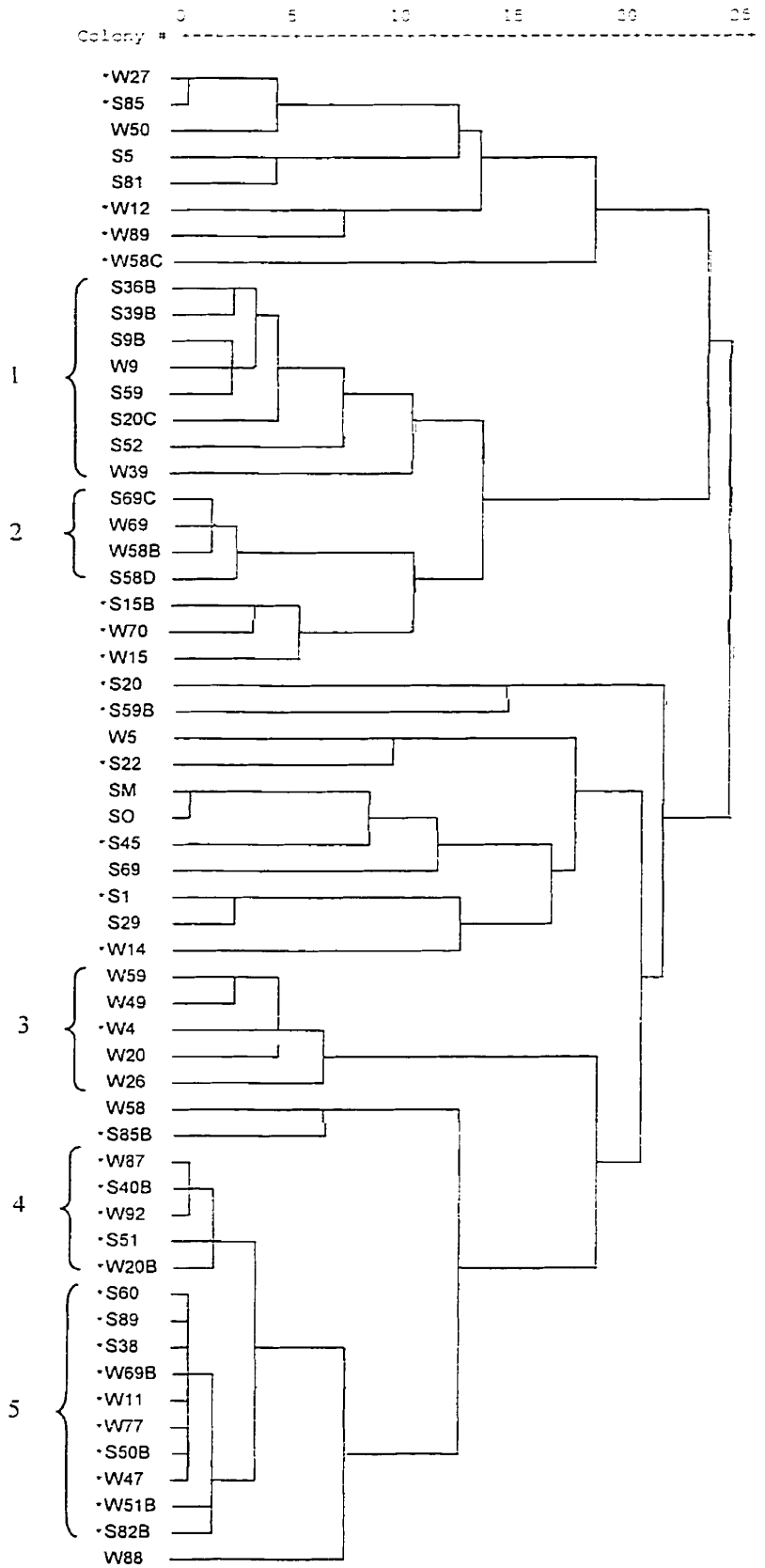

FIG. 2.9. Dendrogram created using cluster analysis with colony identification profiles of S15 and W15 samples. Isolates able to grow at 5°C are marked with an asterisk.

GENERAL CONCLUSIONS

The Biolog system has the potential to be a successful tool in characterizing bacterial communities, but lacks in the concentrations and selection of carbon sources that make up Biolog plates since their combination does not reflect the communities' natural environment. Despite the possibility that high substrate concentrations in Biolog plates may select for fast-growing bacteria (13, 19), CLPPs have shown to reveal temporal or spatial community differences if interpreted with caution (13, 16). Several microbial ecologists also suggest the inoculation of standardized cell densities (7, 11, 30), and short incubation periods to measure community differences due to intrinsic differences in community patterns and not due to the enrichment of dominating species following a lengthy incubation period (13). Community level physiological profiles can be performed at different temperatures, certainly down to 6°C, but extreme caution is needed in their interpretation. Parameters that are read early appear better than those which are read later in the colour development of the Biolog plate. Early readings prevent any complications that arise from the enrichment process in each well and give a better picture of the indigenous bacterial community. Kinetic parameters, lag and slope, do not appear to offer any advantages when dealing with different temperatures. The Arrhenius plots of the substrate colour responses indicate that certain substrates react differently to temperature decreases than others and again, this complicates the interpretation of CLPPs.

The Biolog parameters used in this study (AOD, AWCD, lag and slope) do not pick up a seasonal shifts in the bacterial communities of the Cornwallis River. These

results suggest that these communities have successfully adapted to survive at cold temperatures.

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APPENDIX I

MEANS.XFM

```
;***MEANS AND RANGES FOR BIOLOGS****
;This transform averages two blocks of
;Biolog plate data.  The first block MUST be
;in columns 2 to 13, second block in
;columns 14 to 25.  Column 1 is reserved
;for time of sampling (hours).
;The means are placed in cols 27 to 38,
;ranges in cols 40 to 51.

;******* Data declaration****************
cell(26,1)=" "
cell(39,1)=" "
for n=2 to 13 do
 a=col(n)
 b=col(n+12)
 ;**********Calculations******************
 f1(a,b)=(a+b)/2
 f2(a,b)=abs(a-b)
 mean=f1(a,b)
 range=f2(a,b)
 put mean into col(n+25)
 put range into col(n+38)
end for
```

LOGISTZ.FIT

```
[Parameters] ;4 Parameter Logistic Function
          ;f=(a-d)/[1+(x/c)^b]+d, if b>0 it
          ;starts at a and falls to d.  If b<0
          ;it starts at d and rises to a.

;Modify these values for your data
a=2.0   ;asymptotic maximum
b=-5       ;slope parameter, b>0 gives slope<0
c=60     ;value at inflexion point
;d=.01       ;asymptotic minimum

[Variables]
x=col(1)      ;change to appropriate column
y=col(38)      ;change to appropriate column

[Equations]
f=(a)/(1+(x/c)^b)
          fit f to y
;Five parameter logistic function
;Add parameter e to [Parameters]
;f=(a-d)/(1+(x/c)^b)^e+d
```

PLATES.XFM

```
;*****PARAMETER ESTIMATION FOR BIOLOG PLATES**********
;This transform is to be used on databases containing the two sets of Biolog plate
data plus means, ranges and best fit estimates after using logistz.fit. This will
produce a database with 87 columns. The present transform continues and works
out mu (the growth rate) and lambda (the lag phase) and tabulates it all for export to
SPSS
;******************Identification for SPSS*****************************
cell(90,1)=4          ;person identifier, : Helene=1, Jacob=2, Scott=3
cell(90,2)=7          ;month identifier, change to reflect month of your sample.
cell(90,3)=25          ;day identifier, change to refect day of your sample.
cell(90,4)=8          ;plate row identifier, A=1, B=2, C=3, D=4, E=5, F=6,G=7, H=8.
cell(90,5)=1          ;details of your sample; specifically :-
                      ;for Helene, summer=1, winter=2
                      ;for Jacob, HW/Hppt=1, HW/Lppt=2, LW/Hppt=3, LW/Lppt=4.
                      ;for Scott, crude=1, filtrate=2, retentate=3
cell(90,6)=1          ;invariate, the incrementer for the FOR loop.

**********************Calculations***********************************
for n=0 to 33 step 3 do
 a=cell(52+n,1)
 b=cell(52+n,2)
 c=cell(52+n,3)
 mu=-0.25*((a*b)/c)
 lambda=c*exp(2/b)
 person=cell(90,1)
 month=cell(90,2)
 day=cell(90,3)
 row=cell(90,4)
 detail=cell(90,5)
 well=cell(90,6)
 put person into cell(100,cell(90,6))
 put month into cell(101,cell(90,6))
 put day into cell(102,cell(90,6))
 put row into cell(103,cell(90,6))
 put well into cell(104,cell(90,6))
 put detail into cell(105,cell(90,6))
 put a into cell(106,cell(90,6))
 put b into cell(107,cell(90,6))
 put c into cell(108,cell(90,6))
 put mu into cell(109,cell(90,6))
 put lambda into cell(110,cell(90,6))
 cell(90,6)=cell(90,6)+1
end for
```

PERCHANG.XFM

```
ctrl = col (2)
for n = 2 to 97 do
ref = col (n)
bckgrd = cell (98, n - 1)
f1 (ref,ctrl, bckgrd) = ((ref-ctrl)/ctrl) * 100 - bckgrd
result = f1 (ref,ctrl,bckgrd)
put result into col (97 + n)
end for
cell (189,13) = "    Sum"
cell (189,15) = "    AWPC"
```

ADJUSTOD.XFM

```
ctrl = col (2)
time = col (1)
col (295) = time
for n = 2 to 97 do
ref = col (n)
f1 (ref,ctrl) = (ref-ctrl)
result = f1 (ref,ctrl)
put result into col (294 + n)
end for
```

APPENDIX II

GNCOLOR.24

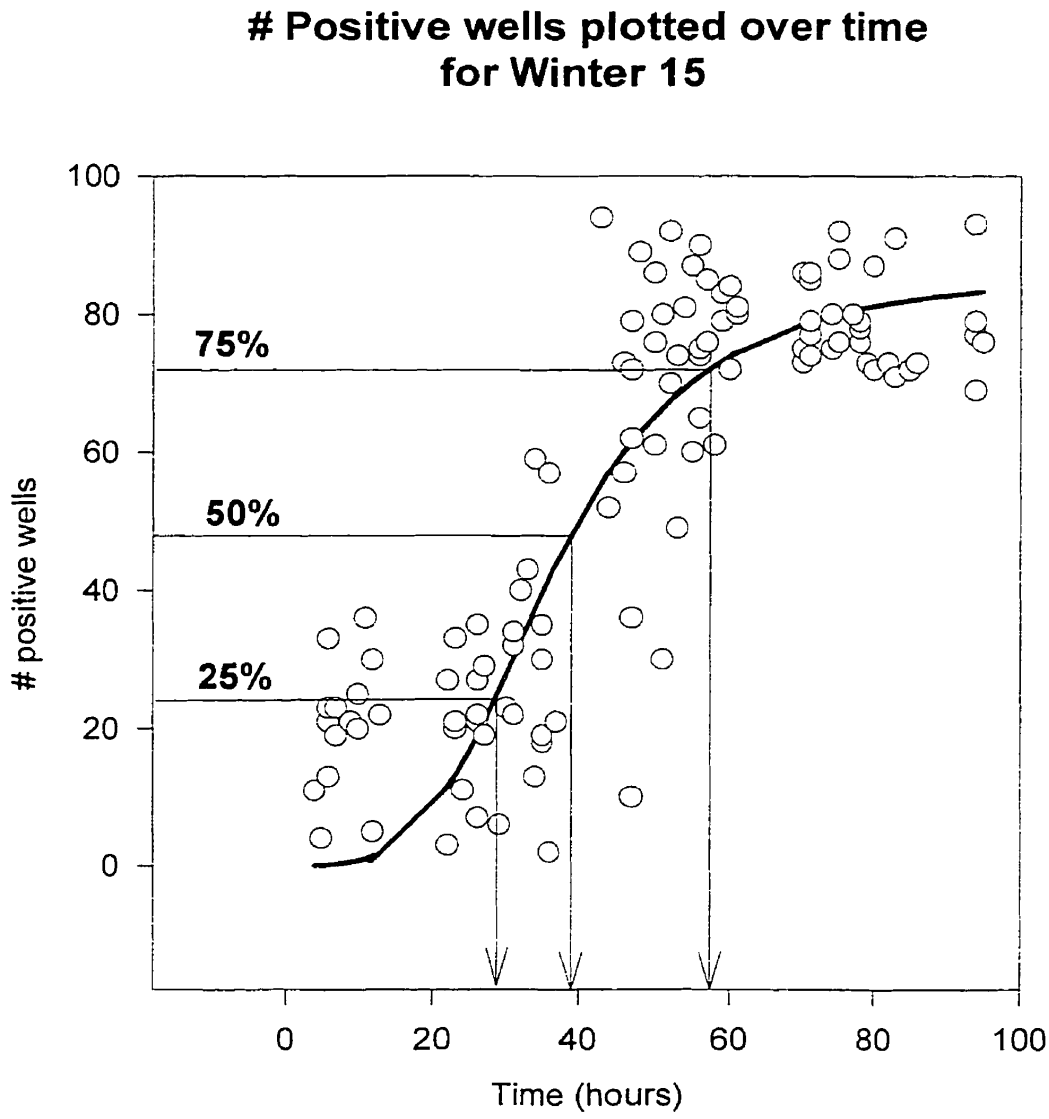
This file is a list of the background percentage correction factors for each well (96 in total); the percentage of the OD reading taken into account by the presence of substrate and tetrazolium dye.

Row 1, column 1

-0.000	5.679	1.556	2.766	0.982
8.597	1.107	1.268	6.283	1.767
7.680	1.201	1.808	-1.068	0.587
10.164	1.003	0.583	2.037	4.148
5.909	-0.813	2.914	0.815	9.203
-1.203	0.038	3.802	1.469	4.686
-1.988	4.584	1.388	1.699	5.382
-0.256	4.125	2.986	1.211	1.627
-3.699	-1.679	-0.036	3.459	-3.464
8.013	3.094	-3.450	4.036	3.202
-0.153	2.179	5.499	2.664	3.856
7.797	0.867	9.522	5.707	4.654
-4.961	5.938	-0.829	6.132	2.535
-0.318	0.292	6.762	-0.388	5.794
-2.067	3.714	-0.736	1.302	5.443
-0.876	11.971	4.133	5.434	9.723
4.886	7.282	-0.107	2.154	row12, column 8
-2.040	-0.554	-0.031	5.139	
-2.366	1.850	-4.145	4.308	
2.945	0.901	8.460	2.000	

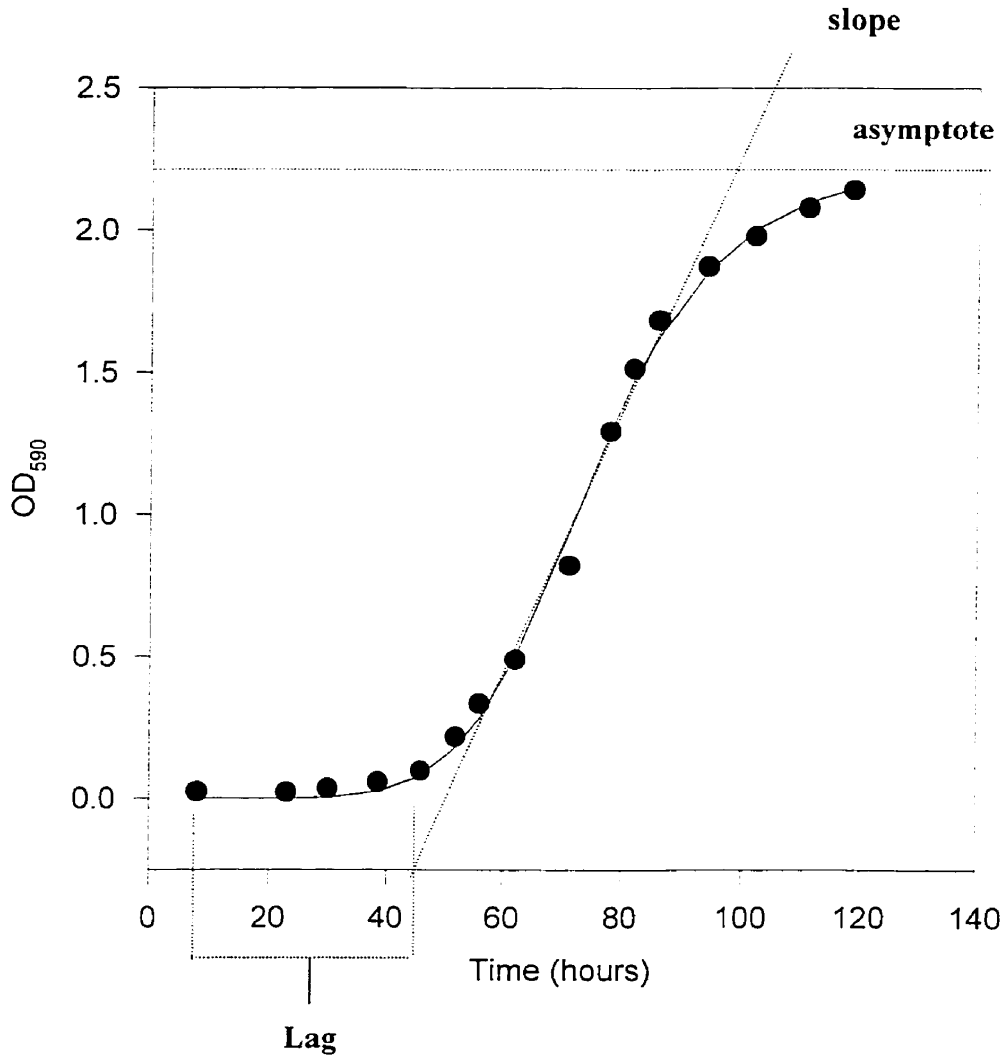
APPENDIX III

Sample graph of how 25%, 50%, and 75% AOD reference times were calculated.



APPENDIX IV

The figure below is a sample of a logistic curve showing the asymptote, slope and lag parameters.



APPENDIX V

1. Thymidine incorporation:

Cells/ml/hr = ((dpm in sample / specific gravity) × (4.5×10^{-13}) × (2×10^{18} cells/mole)

2. Acridine orange direct count:

n = Yad / av = number of cells / ml

Y = mean number of cells / graticule
A = effective area of filtration
d = dilution factor
a = graticule area
v = volume of sample

3. Coliform counts:

(number of colonies) × (dilution factor) / volume filtered = coliforms/ml

4. Spiral plating:

CFU / ml =
(number of colonies in area 1) + (number of colonies in area 2) / area constant

APPENDIX VI

The following is a list of definitions of terms and acronyms utilized throughout this thesis.

AOD	Adjusted Optical Density: AOD is calculated by substracting the raw optical density of the control well from the optical density of each of the remaining wells.
AOD25, 50 and 75	Adjusted Optical Density when 25%, 50% and 75% of the wells have turned positive. A well is considered positive when there is a greater than 40% change in its initial optical density.
Arrhenius plot	The logarith of a rate plotted against the reciprocal of the absolute temperature. The slope of the curve equals the temperature characteristic.
AWCD	Average Well Colour Development. AWCD is equal to the adjusted optical density divided by the 95 wells.
CLPP	Community Level Physiological Profile: The carbon utilization profile of a microbial community using Biolog microplates which contain 95 differenct carbon sources. The oxidative metabolism of the substrates is measured and generates patterns of potential carbon source utilitzation
enrichment	A change in population over time that may occur in any well. Evidence suggests that enrichment causes a decrease in species diversity.
GN microplate	Gram negative Biolog microtitre plate. It is made up of 96 wells, 95 of which contain a carbon substrate and tetrazolium dye. The first well is the control with no carbon substrate. Oxidation of the substrate due to bacterial metabolic processes is coupled with the reduction of tetrazolium dye to produce a purple colour characteristic of the inoculant. The degree of substrate oxidation is determined colormetrically at a wavelength of 590 nm.
SF-N microplate	Used for metabolic testing of sporulating and filamentous microorganisms. It is identical to the GN microplate, but does not contain the tetrazolium redox dye which is toxic to many of these species.

temperature characteristic — It is a measure of the energy of activation of a reaction. It can be calculated using the formula:

$$\text{temperature characteristic} = \frac{2.303RT_1T_2(\log 10rate2 - \log 10rate1)}{T_2 - T_1}$$

PC — Principal Component. The first PC extracted using Principal Components Analysis represents the linear combination of observed variables that maximally separates samples by maximizing the variance of their component scores.

PCA — Principal Components Analysis. A multivariate test which reduces the number of variables to a manageable number. The aim is to identify a small number of linear combinations of the 95 substrates, which account for as high a proportion of the plate-to-plate variation as possible. Prior to the analysis, the raw data is transformed to a correlation matrix and principal components are extracted using these correlations. The loading or score measures the contribution of a variable to the component and these scores are then used to create a two or three-dimensional scatterplot.

The following table lists each parameter used in the analysis of Biolog community level physiological profiles and a brief explanation of each.

parameter	description
AOD	Adjusted Optical Density = OD of the response well – OD of the control well (A1).
	AOD25, 50 and 75 were chosen as 3 reference points for calculating the response of a sample week when 25%, 50% and 75% of the wells in the plate were positive. All 3 reference points represents earlier readings than AWCD 0.5
AWCD 0.5	Average Well Colour Development = [Σ(response well OD – control well OD)] / 95
	An AWCD 0.5 represents a sample with an Average Well Colour Development value of 0.5 absorbance units. This value represents a later reading when greater than 80% of wells have turned positive.
Slope	The tangent to the inflection point
	Slope is a measurement of OD over time. The use of this parameter eliminates having to select the best time to take sample measurements, incorporating early and late readings.
Lag	The x-intercept of the tangent
	Lag phase most likely reflects an adjustment phase during which the community induces the enzymes that are essential for catabolic processes. Lag represents an early reading.