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Methods of Studying Population Shifts in Aquatic Bacteria in Response to Environmental Change

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Maxine A. Holder-Franklin

Scientific Editor
D.B. Carlisle
Research Co-ordination and Program Evaluation Branch

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The study site on the Saint John River, New Brunswick

Environment Canada   Environnement Canada

Methods of Studying Population Shifts in Aquatic Bacteria in Response to Environmental Change

Maxine A. Holder-Franklin*

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Abstract

Population shifts in heterotrophic aquatic bacteria from a watershed with several months of ice cover annually and a temperature range of 0°C to 28°C have been studied by classical and numerical taxonomy, factor analysis and species diversity. Mathematical techniques have been utilized to demonstrate correlations between changes in the bacterial populations and certain physico-chemical environmental parameters. A complete methodology has been developed, beginning with isolation and culturing procedures and proceeding through the taxometrics to the multivariate statistics and the selection of correlation coefficients. This series of reports is a retrospective of work over a period of ten years and describes the seasonal and diurnal changes in the predominant flora in the free-flowing river and the sediment. The major environmental influences in each econiche are related to the bacterial flora.

Although the bacterial population can be shown to fluctuate diurnally and seasonally, the overall predominant taxonomic groups in all locations in all seasons are the Pseudomonas, with the fluorescent group generally predominant in the winter and the non-fluorescent species prevailing during other seasons. The most important group are the gram-negative rods and of these the aerobic group dominate. The aerobes are followed by the facultative aerobes and members of the Enterobacteriaceae appear with moderate frequency in the high-profile segment of the population.

The environmental parameters which have the most significant influence on the flora are the temperature; oxygen levels; the nutrients, particularly the amount and form of nitrogen; and the specific conductivity.

Résumé

L'évolution d'une population bactérienne hétérotrophe vivant dans un bassin versant a été étudiée selon les taxonomies classique et numérique, ainsi que selon l'analyse des facteurs et de la diversité des espèces. La température de l'eau variait de 0°C à 28°C, et les eaux de surface étaient gelées pendant plusieurs mois de l'année. Un processus mathématique a été employé afin de démontrer des corrélations existant entre les variations d'une population bactérienne et certains paramètres physico-chimiques de l'environnement. Une méthodologie complète a été élaborée, commençant par les procédés d'isolement et de cultures, passant ensuite aux statistiques taxométriques et multivariées et à la sélection de coefficients corrélatifs. Les recherches ont été conduites au cours d'une période de dix ans. Le présent rapport décrit les changements quotidiens et saisonniers de la flore bactérienne prédominante dans le courant du fleuve et dans les sédiments. Les influences les plus importantes de l'environnement sur chaque niche écologique sont liées à la flore bactérienne.

Bien que la population bactérienne fasse état de fluctuations quotidiennes et saisonnières, les groupes taxonomiques qui prédominent en général, en tout lieu et saison, sont les Pseudomonas. Dans ce groupe-ci, les fluorescents sont généralement prédominants l'hiver, alors que les non-fluorescents sont majoritaires durant les autres saisons. Les bâtonnets de gram négatif forment le groupe le plus important. Dans ce dernier groupe, les aérobies sont dominants, puis viennent les aérobies facultatifs et quelques Entérobactériaceae.

Les paramètres de l'environnement qui ont l'influence la plus significative sur la flore sont: la température; la quantité d'oxygène; les aliments, notamment la quantité et la conformation d'azote; et la conductibilité spécifique.

Acknowledgments

The authors wish to acknowledge the contributions to the development of this work of Dr. W. E. Lowe, who as the Head of the Research Subventions Program strongly supported this program and also as a microbiologist acted as a valuable consultant and devil's advocate. We would also thank Dr. David Carlisle for his support, intellectual stimulation, and more recently his patience, in making this publication possible. Over the years many diligent and tenacious workers have made significant contributions to the final fruition of the project: our colleagues Lawrence Wuest and Jane Fritch, mathematicians and programmers, students Marjorie Chalifour, Clarence Cormier and Alan Thorpe; postdoctoral fellows Colin Bell and Tatsuo Kaneko, photographers Roger Smith and Terry Edwards, graphic artist Americo Buzzeo, technicians Sharon Kennedy, Linda Merry, Noreen Hood, Freda Byno, Colleen Curry, Teiko Kaneko and Joseph McCulley, and secretaries Sharon Lutwick and Shirley Pullen; the final document was processed by Veronica Burleigh and Lucia A. Brown and we are grateful for their great efficiency and skill.

A special thank you to Dr. R. R. Colwell, who encouraged us to use numerical taxonomy.

This work was supported by grants from the Research Subventions programme of the Inland Waters Directorate, Department of the Environment, and the National Research Council.

Summary

Population shifts in heterotrophic aquatic bacteria in the Saint John River system have been studied by classical and numerical taxonomy using selected physiological responses, and the mathematical analyses - factor analysis, correlation procedures and species diversity. Ecological relationships between the environment and the bacterial flora have been established where possible.

The Saint John River is 450 miles long; its headwaters are in northern Maine and the river forms the international boundary for approximately 100 miles. The watershed is shared between the state of Maine and the province of New Brunswick.

The microbial ecology of rivers in the temperate zone has received very little attention. In the northern part of this region the climate ranges from subtropical heat and heavy rainfall to sub-zero temperatures with several months of snow annually and considerable ice cover. The water temperature ranges from 0°C to 28°C. When these studies were commenced in 1970, it was necessary to design a new methodology to determine the heterotrophic bacterial flora in the water, to detect changes in the flora and to assess the significance of the changes.

A methodology has been developed which utilizes an extensive numerical taxonomy data base to profile the population of heterotrophic bacteria in the midstream subsurface area of the river. The predominant population was classified as to genus and species. The data base, in the form of test responses of individual isolates, is then factor analyzed. This multivariate technique compresses the variance and forms hypothetical constructs which can be interpreted biologically. Each important biological feature of the population is represented by a dimension which can be compared with various environmental chemical and physical parameters. The factor analysis reveals the essential character of the population so that mathematical correlations can be made with the environmental parameters, thus demonstrating that the environmental change has produced a corresponding change in the bacterial population.

As the studies progressed, it became increasingly more evident that microbiological data from natural sources has little significance unless it is interpreted within the context of the chemical and physical character of the environment. This series of papers reports one phase of the studies on the Saint John River and its tributaries, the Meduxnekeag and the Dunbar, and is presented as a retrospective to elucidate the development of the methodology and how that methodology was utilized in an ecological study.

The taxonomic studies were commenced at the Florenceville site above and below the McCain food processing plant, which produced frozen vegetables at the time of the study. The preliminary study provided information on the types of bacteria present, showed that seasonal changes in the populations could be observed, and that special vitamins and growth factors were required by the summer isolates. Armed with this information, the first studies on numerical taxonomy could be more carefully designed. The selection of the test base was a critical feature of planning, particularly in these studies where the objectives had been broadened to include a wide range of heterotrophic species.

Both studies indicated that the bacterial populations above and below the plant were similar and that seasonal changes in taxa and physiological functions of the organisms occurred. The plant effluent organisms isolated from the point source were quite different from the river bacteria one mile upstream or downstream and it was concluded that these organisms became diluted out and did not become established in the econiche under study, the free-flowing river. Throughout the study, organisms which clustered were considered to be the predominant group, having an ecological advantage to achieve the numbers required for detection by the methods used. These clustered isolates were identified by the classical methods, phenotypic traits and DNA analysis.

The genera which predominated most frequently in all studies were the Pseudomonas, Aeromonas and Flavobacterium. The Cytophaga and Flexibacter were of secondary importance and some fermentative types appeared usually in the warmer months. The one striking feature of the bacterial population was the predominance of Pseudomonas fluorescens in the winter samples. In the Dunbar, in September and October Pseudomonas fluorescens predominated in both water and sediment, however, in August P. fluorescens predominated in the water only and was not detected in the sediment. Although the evidence suggested some seeding of the water from sediment populations in the summer in the Meduxnekeag and in the autumn in the Dunbar, the overall factor analysis suggested two very distinct populations in these two econiches.

Fermentative organisms were observed in higher numbers and more frequently in the summer samples. The river population in general was composed of aerobic, mesophilic, non-fermentative rods. Temperature was the most important controlling factor - near-freezing water favoured the Pseudomonas, however, many of the winter organisms were facultatively psychrophilic and most accommodated wide temperature ranges. Some seasonal influence was observed and the numbers of organisms that grew at higher temperatures did increase in the summer. The number of organisms that grew at less than 10°C did increase somewhat in the winter. The majority of organisms isolated winter and summer can grow at temperatures from 10°C to 43°C. The numbers of obligate

psychrophiles were small even in the winter. Other parameters such as oxygen, nutrient levels and ion concentration were also important features of the controlling environment.

Although the clusters formed in the association coefficient matrix were mathematically homogeneous, the appearance of other species in the clusters did occur. Another problem with the method was the difficulty in separating some highly related Pseudomonas strains into distinctive subclusters. The reference strains clustered with themselves, rarely with the river strains. Improvements were made by utilizing a more stringent clustering procedure.

The first study on the Meduxnekeag where seasonal and diurnal samples were obtained demonstrated the sensitivity of the predominant bacterial population to environmental changes. The objective was to refine the method so that small changes in the flora could be realistically evaluated.

This was accomplished by the demonstration of diurnal variation in the month of October in samples from the Meduxnekeag River. Although the numerical taxonomy data does show this feature, the graphical projections of factor scores as shown in spire diagrams does demonstrate the diurnal changes more clearly. The bacterial populations in the Meduxnekeag were also shown to vary seasonally.

Where possible, each clustered strain in the sixteen seasonal and diurnal samples were speciated. In some of the larger clusters closely related species were observed as subclusters within the larger cluster. Strains within the subclusters were grouped according to sampling time. In the Aeromonas clusters in the September samples from the Meduxnekeag phenotypic differences were observed between one subcluster and another; some of these differences would change the biotype designation. In September and October, the temperature of the water was the same and the samples were taken two weeks apart, however, the bacterial populations were very different. Aeromonas predominated in September with little diurnal variation, whereas Pseudomonas species were clearly dominant in October. The environmental influences of oxygen and ionic concentration have been reported previously to be important controlling factors in the organisms in the Meduxnekeag.

Diurnal variation in bacterial species was slight in the Meduxnekeag and Dunbar river water, however, the sediment populations shifted between the two major segments, the obligate aerobes and the fermentors. The sediment showed a higher incidence of enteric bacteria. This group also exhibited a higher frequency of proteolysis, lipolysis, fermentation, gas production and NO_3 reduction. The water bacteria were more nutritionally versatile, metabolizing more substrates as sole carbon sources. Nitrate was a major physico-chemical parameter affecting the water bacteria. Using a wide range of criteria to study the interactions of sediment and water, it was concluded that the interactions were negligible.

The most significant of the limitations of the method is the necessity to produce a viable isolate. Also, the isolate must be examined after minimal manipulation; repeat tests weaken the conclusions on the original state of the organisms; and many organisms in the samples will not propagate on laboratory media. Even with these limitations it has been possible to obtain profiles of the heterotrophic bacteria which clearly correlate with the physico-chemical condition of the water. The differences observed between the three rivers underscore the dangers of relating observations in shallow highly aerobic pristine streams to silty-bottom polluted deep rivers.

Some surprises awaited. At the outset, we expected a very sluggish bacterial population to be hibernating under the ice and observed metabolic versatility. We expected Pseudomonas fluorescens in the Meduxnekeag in September at 12°C and isolated Aeromonas and Flavobacterium with an unusually high specific conductivity in the water. We were convinced that we would observe a very active interchange between the sediment and the water in the Dunbar and found the reverse. We expected that this research would provide some answers and instead discovered more questions.

Introduction

From 1970 to 1980, a group of researchers at the Microbiology Research Laboratory, University of New Brunswick, (Annex C) were engaged in a series of physiological, taxonomic and statistical studies to determine the types and activities of bacteria in three New Brunswick rivers. The observations ranged over responses of the bacterial population in batch and chemostat cultures to variations in substrates, and changes in populations in response to chemical and physical changes in the environment using statistical methods and computerized analysis. This collection of papers will describe the preliminary studies in taxonomy, the development of the methodology to study population shifts and the conclusions formed on the responses of the bacterial population to environmental changes.

Samples were taken from several locations on the Saint John River and its tributaries from 1970 to 1978. The basin of the Saint John River is a mixture of woodlands and cleared farmlands with small communities dotting the shores. The main stream of the river is primarily a recreation area with several dams and one major impoundment which has widened the river for 160 km upstream. Over the years, the banks have expanded and the water level has increased. Only one site near this impoundment has been chosen. Most of the sample sites are in narrower free-flowing areas of the rivers, and one stream in particular, the Dunbar, was chosen for its pristine watershed for comparison purposes. One site was located near an obvious pollution out flow, a food processing plant in Florenceville, New Brunswick. The seasonal changes in the sampling area were similar. The map shows the sampling sites and approximate distances (Plate 1).

The first report describes the preliminary stage in which the flora near the McCain food processing plant (Florenceville) was first isolated. At this stage, we did not know what organisms to expect as there were very few reports in the literature in 1970.

The main body of the research reported here centres around the development of methods to analyze changes in the bacterial population of fresh water in response to environmental changes. The profile of the population was obtained first from numerical taxonomy and then from a multivariate analysis of the test response data base, followed by a correlation of the biological profile with the environmental profile. The methodology was successful in relating these two complex data sets. Using mathematical techniques, the enormous variation and complicated interrelationships of upward of 200 primarily physiological and nutritional test responses and 19 chemical and physical parameters have been reduced to a manageable analysis and a logical interpretation of the results. A substantial amount of the data collected over the years has been presented or is in the process of being presented in other publications. This report contains a retrospective of the development of the methods using certain examples. A detailed account of the more complex mathematical methods has not been included.

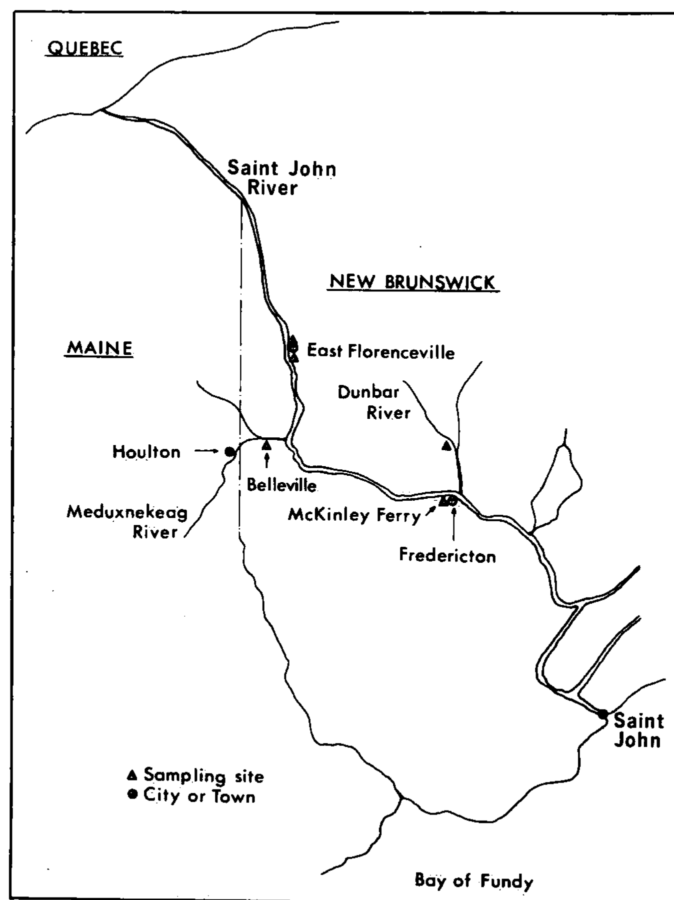


Plate 1. Map of Southwestern New Brunswick showing sampling sites.

Methodology of Isolation and Identification of Heterotrophic Water Bacteria with a Detailed Description of Numerical Taxonomy

M.A. Holder-Franklin, T. Kaneko, M. Franklin, L.J. Wuest and C. Cormier

Sampling Methods

Water samples were obtained by two methods:- by immersing a sterile plastic bottle just under the surface and by tapping the sampling port in the monitoring station maintained by Environment Canada, Water Quality Branch, at Belleville, New Brunswick. Water is continuously pumped through the system in the monitoring station where probes detect hourly changes in several physical and chemical parameters and feed the information to the computer terminal in the station. The data is then stored on tape (Plate 2).

Water samples collected manually were transported immediately to the laboratory for dilution and plating.

Microbiological Analysis

Each sample consisted of two 1000-ml Nalgene screw cap bottles filled and plated at the field monitor station according to the following procedure. Immediately prior to diluting for plate counts, 350 ml from each sample was mixed in a Sorvall Omnimixer Model No. 17150 at setting No. 4 for four minutes. High speed mixing in an omnimixer serves to break up chains or clumps of cells without killing the bacteria, thereby giving a more accurate count in the number of viable bacteria (Slade & Slamp, 1956). Each of the duplicate samples was diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} (again in duplicate) in sterile distilled water. From each dilution blank, 0.1 ml was plated onto the surface of casein-peptone-starch (CPS) medium (Appendix B) Collins & Willoughby (1962). The inoculated plates were incubated in a Hotpack Refrigerated Incubator, Model 352700 at 20°C for 10-14 days and the numbers of viable bacteria per millilitre were determined. Individual colonies were selected at random from plates with 100-150 colonies and subcultured on CPS three times to ensure purity of the cultures (Stanier et al. 1966).

Cultures were maintained on Lowe Agar (Appendix B).

All manipulations of cultures were performed in a laminar flow hood (Envirogard).

Reference strains used in these studies were treated in a manner similar to fresh isolates and were tested simultaneously as a quality control procedure. A list of these strains and their sources are shown in Appendix A.

Liquid Nitrogen Preservation

A. Thorpe and M. A. Holder-Franklin

Studies of aquatic bacteria require examination of the characteristics of several hundred isolates simultaneously. An essential prerequisite is the preservation of such strains in a form which corresponds closely to the natural state with a minimum of manipulation and a high degree of viability on recovery.

A method of preservation was required which would meet the following criteria: that (a) large numbers of fresh isolates could be prepared rapidly, (b) storage containers be compact and moveable and (c) regenerated isolates have a high level of viability without repeated transfer. Freezing the organisms in liquid nitrogen met these criteria.

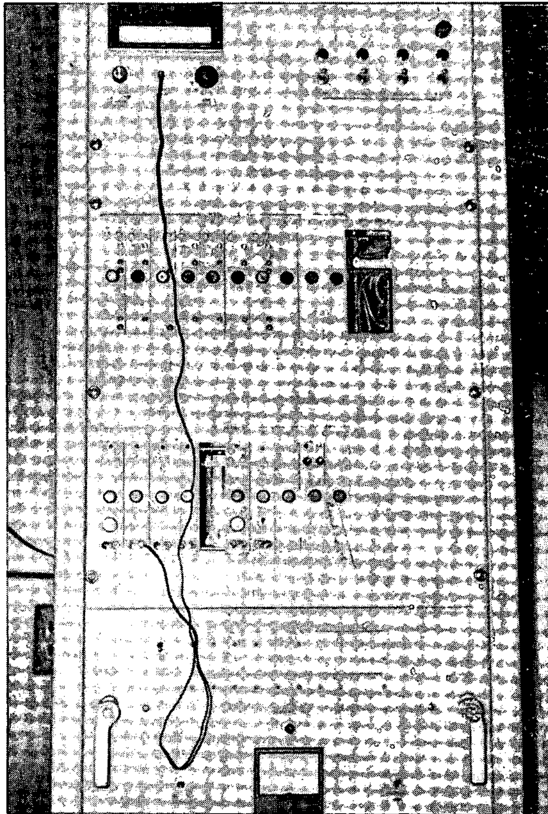
Microorganisms may be frozen by slow cooling at a rate of 1°C to 10°C/min (Araki, 1969) or rapid freezing by plunging the cultures into coolant at temperatures ranging from -60°C to -195°C (Smith, 1973). Rates of cooling of less than 10°C/min do not result in a significant loss of viability. There is a direct relationship between the loss of viability and the increase in the cooling rate (Nei et al. 1969). According to Mazur (1963), cells which survive the initial cooling to -50°C followed by an immediate return to room temperature will survive indefinitely if the storage temperature is less than -100°C.

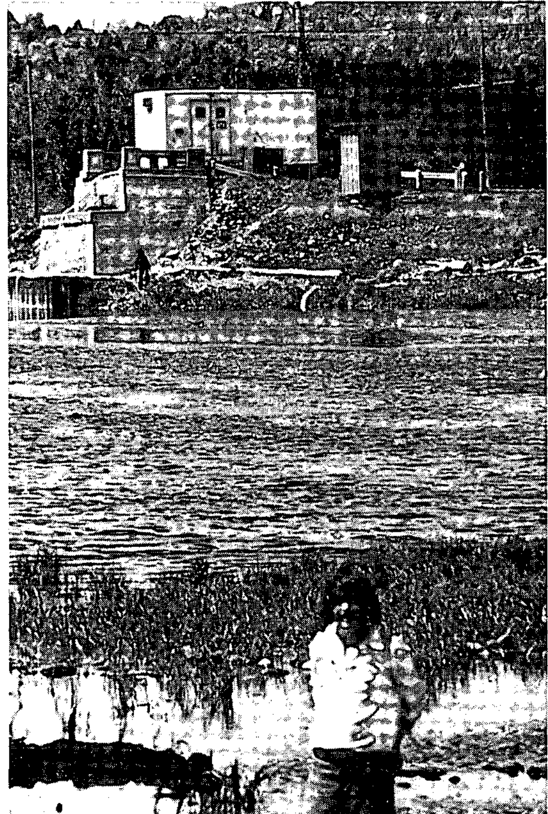
One of the most serious drawbacks to freezing is severe cell injury, which can be mitigated by the use of cryoprotective agents such as glycerol or dimethyl sulfoxide (DMSO)(Moss & Speck, 1966; Szmant, 1969). The injury is due to the loss of enzyme activity and damage to the DNA. However, trypticase soy is an effective restorative agent and repair occurs within a few hours.

There are several reports of successful long-term storage of bacteria in the frozen state. In one laboratory, bacteria have been preserved in liquid N_2 for nine years and it has been predicted that these strains will remain viable indefinitely (Nei et al. 1969).

METHOD

Pure cultures of bacteria were transferred to Lowe agar slants for seven days at 20°C. The bacterial growth from two slants was suspended in 2.0 ml of trypticase soy broth (BBL) and one of the preservation agents - 7.5% D-glucose, 7.5% DMSO or 10% glycerol - was added . Immediately following vigorous shaking in a vortex





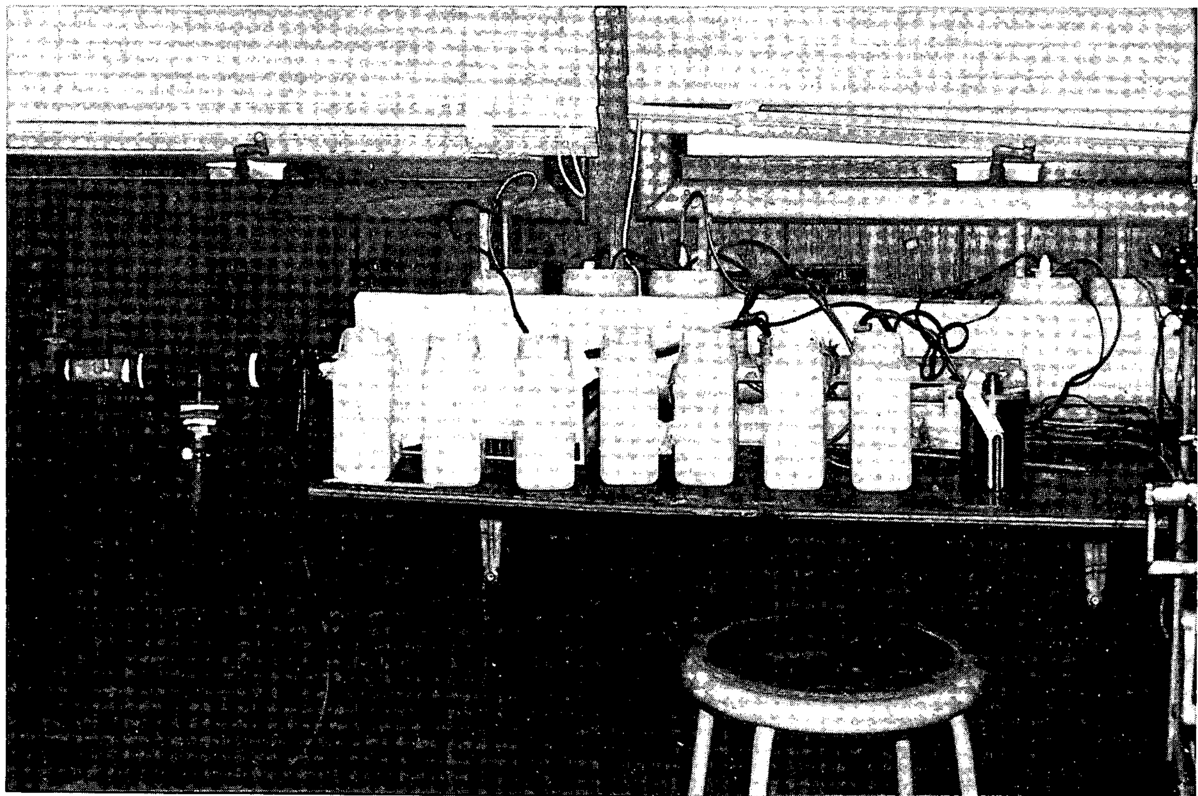


Plate 2. (left) Computer terminal (right) Belleville monitoring station (bottom) Sampling device - probes, sampling bottles, omnimixer container

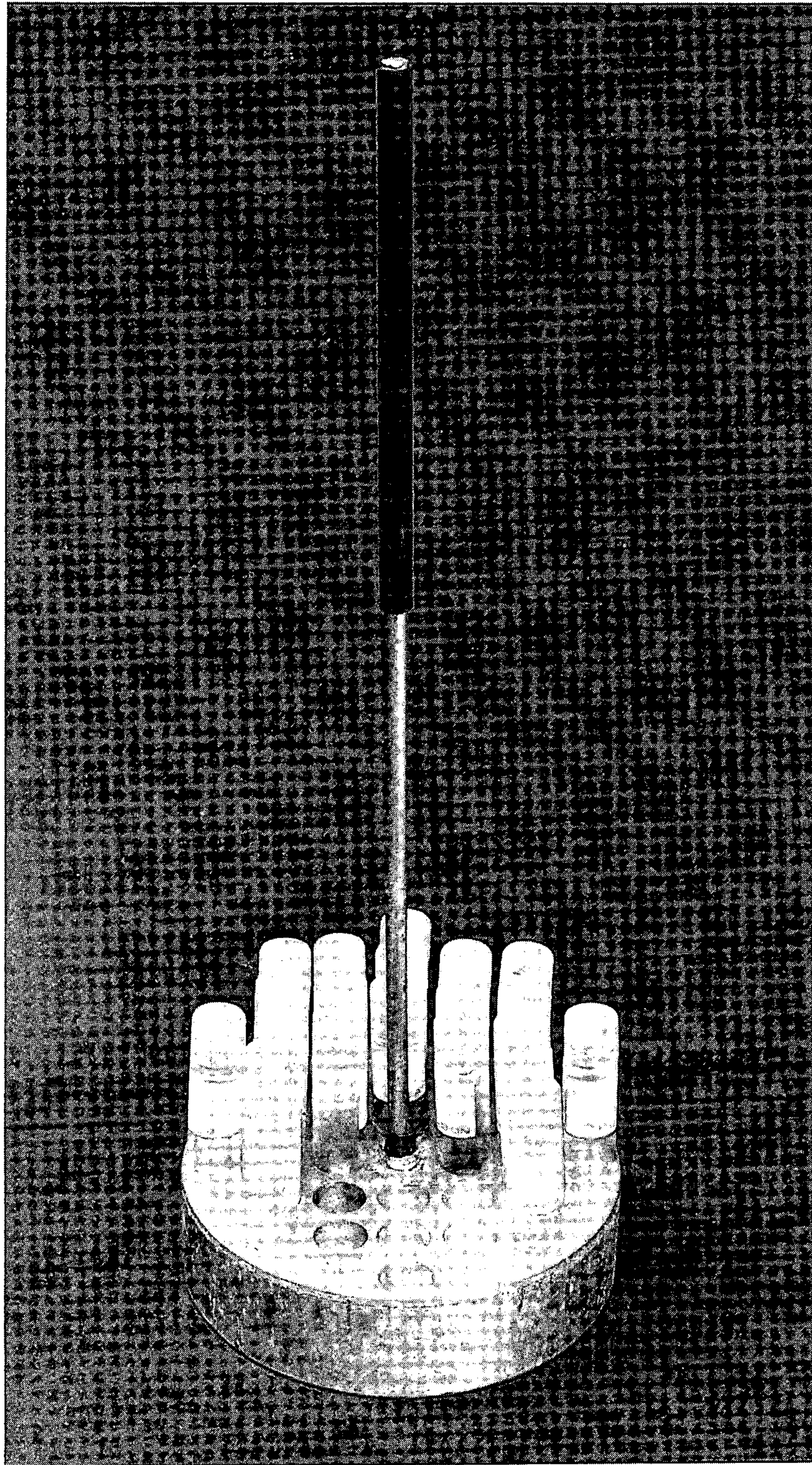


Plate 3. Holder for vials for freezing bacteria. Material: Base Aluminum Alloy, 29 holes at regularly spaced intervals. Handle: Standard Inoculating Loop Holder will accommodate standard 1- or 2-ml glass or plastic ampoules.

mixer, 0.5 ml aliquots were placed in four sterile plastic vials (Union Carbide 30 x 1.2 mm) or 1 ml glass ampoules (Wheaton Glass Co. type 1, 1 ml; gold band). The glass ampoules were labelled with a tungsten carbide tipped electric engraving tool (Model V30/V40, Burgess Vibrocrafters Inc.) and placed in an aluminum holder (Plate 3). The dimensions of the holder are shown in Figure 1 (overall diameter 10 cm). The holder was lowered into the freezing compartment of a Virtis freeze dryer (Model no. 10-010), which was 2/3 full of 95% ethanol at -60°C, for 20 min.

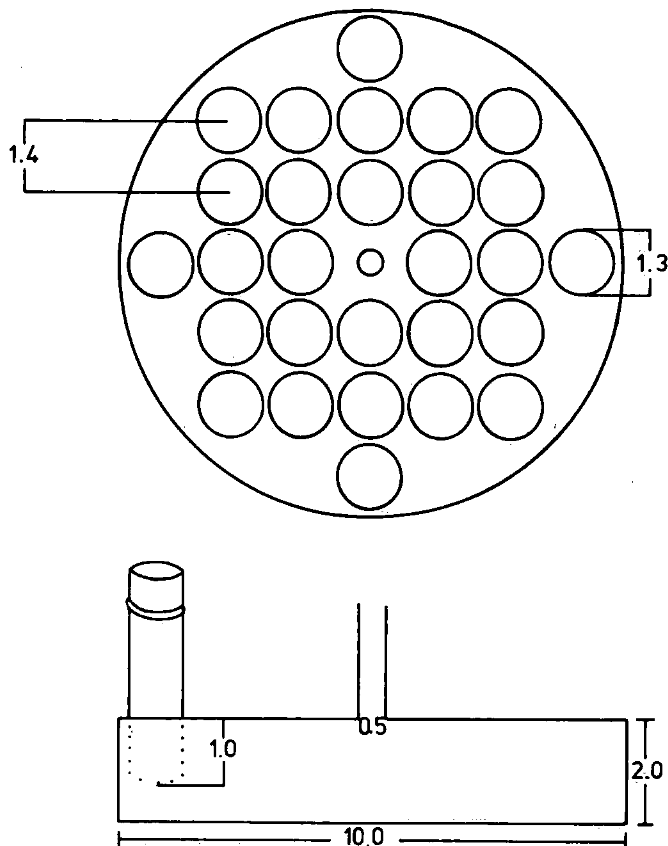


Figure 1. Diagram of tube holder for liquid N_2 freezing (dimensions of holder in centimetres)

The glass ampoules were removed individually from the ethanol and the opening flame-sealed. The ends were further sealed by epoxy glue to ensure that liquid N_2 did not enter the vial. A glass ampoule will explode on warming if N_2 has entered.

The vials were replaced in the ethanol until the sealing and subsequent cooling of the container and contents was completed. They were then placed in Dewar flasks containing liquid nitrogen.

An inexpensive alternative method for controlled freezing in ethanol has been described by Ryan (1974) for use when a freeze-drying apparatus is not available.

Tests for cell injury

Various suspending media, 7.5% D-glucose, 7.5% dimethyl sulfoxide and 10% glycerol were tested for degree of cell injury using the two-step freezing technique. In addition, cells suspended in 10% glycerol were tested for direct freezing in liquid N_2. After freezing for one month some cells were plated onto a minimal medium and others onto trypticase soy agar to determine the number of injured cells. The degree of cell injury can be determined by a comparison of growth on minimal medium with the growth on the enriched medium. The injured cell is one which has not lost viability, i.e. which grows on trypticase soy medium but not on minimal medium. Straka & Stokes (1959), after noting the change in nutritional requirements of frozen cells, have demonstrated that metabolic injury is the most significant damaging factor. It has been shown by several workers that repair and recovery are possible in a suspended medium containing extra nutrients. The minimal medium contained K_2HPO_4 (2.5 g), $MgSO_4$ (0.1 g), NH_4SO_4 (1.0 g), sodium citrate (0.1 g), glucose (2.5 g) sterilized by filtration, agar (15 g) and distilled H_2O (1000 ml). The restorative viable counts (cfu/ml) of the strains grown on trypticase soy agar before freezing were compared with the counts on trypticase soy after freezing and thawing and on minimal agar after freezing and thawing in the various preservation fluids (Table 1). The difference between growth on the enriched medium and growth in the minimal medium indicated the number of impaired or injured cells. This procedure was utilized to determine the best suspending agent for aquatic bacteria.

Rates of cooling comparison

In order to determine if the use of a large holder containing several vials would affect the rate of cooling, temperature readings from 0°C to -40°C were obtained in ethanol using a copper-constantine thermocouple with the reference junction at 0°C. As shown in Figure 2, plastic vials in the holder were cooled at the same rate in ethanol as one glass vial which was not suspended in the holder.

In liquid N_2, temperature decreases were recorded from -40°C to -192°C. The rate of cooling in glass and plastic vials, in the holder, were compared and after two minutes both had reached the lowest temperature which we were able to record (Fig. 3).

As shown in Figures 2 and 3, the cooling curves are similar for glass and plastic vials and for vials immersed in the cooling agent within the holder or held singly. Figure 2 demonstrates that the temperature decreased at the same rate in glass or plastic vials. If cooling in glass and plastic vials was compared during the first two minutes, the contents of the glass vials cooled more rapidly than the contents of the plastic vials. No decrease in viability was observed in cultures frozen in the glass vials.

Viability and injury comparisons

Using a graduated cooling method, first to -40°C in ethanol and then to -192°C in liquid N_2, three suspending media were compared as shown in Table 1. Representative strains isolated from the Meduxnekeag River were plated on trypticase soy and minimal agar, as described. The numbers of viable organisms expressed as

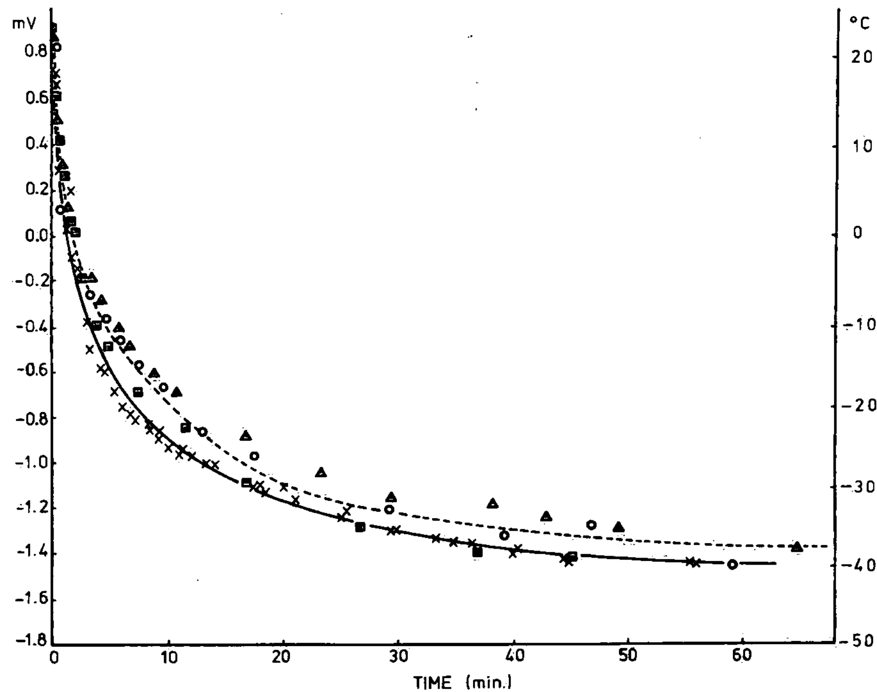


Figure 2. Cooling curve in first-phase freezing.

▲ 1 glass vial + holder
■ 1 glass vial only
○ 12 glass vials + holder
x 3 plastic vials + holder

Table 1. Freezing damage to cells expressed as the percentage of cfu/ml after freezing - compared with counts before freezing.

	Ethanol plus liquid nitrogen						Liquid N_2 Only	
	7.5% Dextrose		7.5% Dimethyl Sulfoxide		10% Glycerol		10% Glycerol	
Isolate Number	% Viable	% * Viable Injured	% Viable	% * Viable Injured	% Viable	% * Viable Injured	% Viable	% * Viable Injured
1	36	83	47	77	45	43	0.02	100
2	67	1	19	100	64	3	0.2	100
3	81	78	40	100	74	58	2.9	98
4	81	67	3	59	84	45	4	100
5	12	64	4	87	14	58	1.5	2.4
6	79	92	10	87	93	2	0.5	66.7
7	94	6	4	65	85	35	6.6	31

* % of the viable cells which demonstrated injury

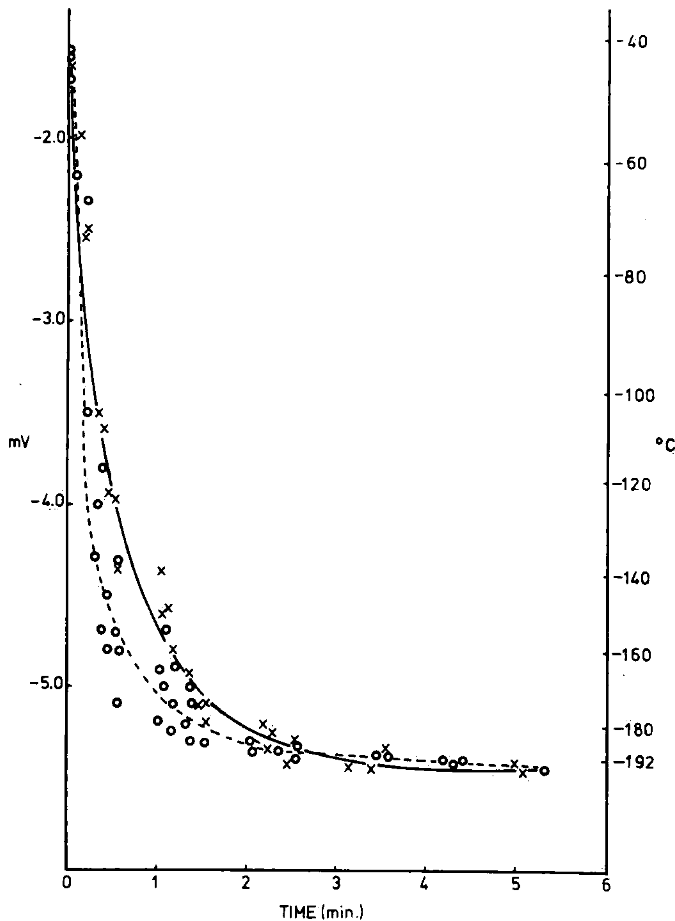


Figure 3. Cooling curve in second-phase freezing (liquid N_2) -40°C to -192°C. Temperature readings measured using a Copper-Constantine Thermocouple with the reference junction at 0°C.

o Glass vials

x Plastic vials

colony forming units/ml (cfu/ml) on trypticase soy and minimal medium were determined before the cell suspensions were frozen.

It is clear from the table that cells frozen in 10% glycerol, using a two-step method, have the highest overall viability and less chance of being injured. Freezing directly in liquid N_2 was contraindicated for these bacteria.

Freshly isolated heterotrophic river bacteria may be successfully frozen by a two-phase process, first to -40°C followed by freezing at -192°C (-196°C, theoretically), and have an improved viability if suspended in 10% glycerol during the freezing process. Recovery of cells should be made on an enriched medium, such as trypticase soy, in order to restore viability and repair injury.

All of the bacterial strains isolated from aquatic habitats and preserved by this method have been fully viable after one year in liquid nitrogen. The trypticase medium has proven to be most appropriate for restoration. This was also true for those freshly isolated aquatic bacteria which grow poorly on enriched media.

Preparation of cultures for testing

T. Kaneko and M. J. Chalifour

Isolates were again inoculated onto Lowe plates and incubated for 3 to 7 days. Suspensions of bacteria, 1 loopful of growth mixed thoroughly with 3 ml of distilled water were drawn into 3-ml syringes and inserted into holes in the multipoint inoculators - one for plates (See Plate 4) (Kaneko et al., 1976) and one for tubes (See Plate 5) (Thorpe, 1979) - developed for this purpose in this laboratory by T. Kaneko.

Taxonomic Tests: - Substrate Utilization Tests

Substrates as listed below in concentrations of 0.1-0.15% were added to the basal medium of Stanier et al. (1966), Appendix B.

Substrates:

D-arabinose	sucrose
L-arabinose	D-xylose
cellobiose	gluconate
D-fucose	inulin
L-fucose	valerate
L-lyxose	isovalerate
maltose	caproate
D-mannose	2-ketogluconate
melibiose	mucate
D-melezitose	saccharate
acetate	salicin
propionate	carboxymethylcellulose
butyrate	trehalose
D-rhaffinose	dihydroxyacetone
L-rhamnose	D-galacturonic acid
D-ribose	alpha-methyl-D-glucoside
L-sorbose	caprylate
starch	pelargonate

.....

oxalate	glutarate
malonate	adipate
succinate	pimelate
maleate	sebacate
fumarate	

.....

DL-malate	DL-beta-hydroxybutyrate
D-tartrate	DL-lactate
L-tartrate	DL-glycerate

.....

citrate	cis-aconitate
alpha-keto-glutarate	laevulinate
pyruvate	transaconitate

.....

erythritol
mannitol
sorbitol
meso-inositol
adonitol
glycerol
ethylene glycol
propylene glycol
D-arabitol
L-arabitol
dulcitol
phenethyl alcohol
arbutin

. . . . .

methanol
ethanol
propanol
n-propanol
benzoate
m-hydroxy-benzoate
3, 4-dihydroxybenzoate
isopropanol
n-butanol
p-hydroxybenzoate
phenyl-1,2-ethandiol
phenol
tertiary-butanol
2-amino-2-methyl-propanol
xylene
ferulic acid

. . . . .

glycine
β-alanine
DL-serine
L-threonine
L-leucine
L-isoleucine
L-valine
L-aspartate
L-glutamate
L-lysine
DL-arginine
L-ornithine
L-methionine
L-cystine
asparagine
DL-α-methylglutamic acid

. . . . .

L-histidine
L-proline
L-tyrosine
L-phenylalanine
L-trytophan
kynurenate

. . . . .

ethanolamine
benzylamine
putrescine
spermidine phosphate
histamine
tryptamine
alpha-amylamine
N-acetylglucosamine
p-phenyl-ethylamine
aniline

. . . . .

betaine
sarcosine
creatine
hippurate
acetamide
DL-carnithine HCL
urea
alantoin

. . . . .

guanine
nicotinate
L-ascorbic acid
uracil
pyridoxine
thiamine HCL
xanthine
pantothenate

. . . . .

deoxycholic acid

. . . . .

lauric acid

Commercially Prepared Agar:

Brain Heart Infusion (BHI) and 0.1 strength BHI, Levine Eosin Methylene Blue (LEMB), MacConkey, Nutrient and 0.1 strength Nutrient, Pseudomonas, Phenylalanine, Simmons Citrate, Sellers Differential, Typticase Glucose Extract, Triple Sugar Iron, Todd Hewitt, Pseudo, (Difco). Pseudosel, Potato Dextrose, Trypticase Soy (TSA), 0.1 strength TSA, Thiosulphate-Citrate-Bile salts-Sucrose, (TCBS)(BBL). Sabaraud Dextrose, SIM (Fisher Scientific).

The results of growth on the commercial agars listed were included in the analysis to establish efficacy of use for future testing; incubated at 20°C for 10-12 days.

Tolerance Tests: - Reagents listed in Appendix C

Bile and Brilliant Green - Miller & Banwart, 1965

Bile salts - Rhodes, 1959

NaCl - Rhodes, 1959; Hayward & Hodgekiss, 1961; Bachman, 1955.

Dyes - Starks & England, 1933; Cruikshank, 1948

Ethanol - Steel, 1957

KCN - Malek et al. 1963

Levan - Klinge, 1960

Phenol - Sneath, 1956

Sodium selenite - Hendrickson et al. 1934

Teepol - Gunther & White, 1961

Tellurite - Whittenbury, 1965

Tetrazolium Salt - Davis & Park, 1962

Miscellaneous Tests: - Reagents, Appendix C

Fluorescent pigments, Phenazine pigments - King et al. 1954

Gelatin hydrolysis - Skerman, 1959

Tween hydrolysis - Sierra, 1957

Starch hydrolysis, tributyrin hydrolysis - Rhodes, 1959

Phosphatase - Barber & Kuper, 1951

Poly-beta-hydroxybutyrate staining - Burdon, 1946, Franklin & Franklin, 1971

Arginine dihydrolase - Thornley, 1960

Denitrification - Stanier et al., 966

Oxidation/Fermentation - Hugh & Liefson, 1953

Tests from Manual of Microbiological Methods (Conn et al., 1957) include:

Catalase, Cytochrome oxidase, H_2S from Cysteine, H_2S from Sodium thiosulfate, Indol, Nitrate and Nitrite reduction, Methyl red, Voges-Proskauer.

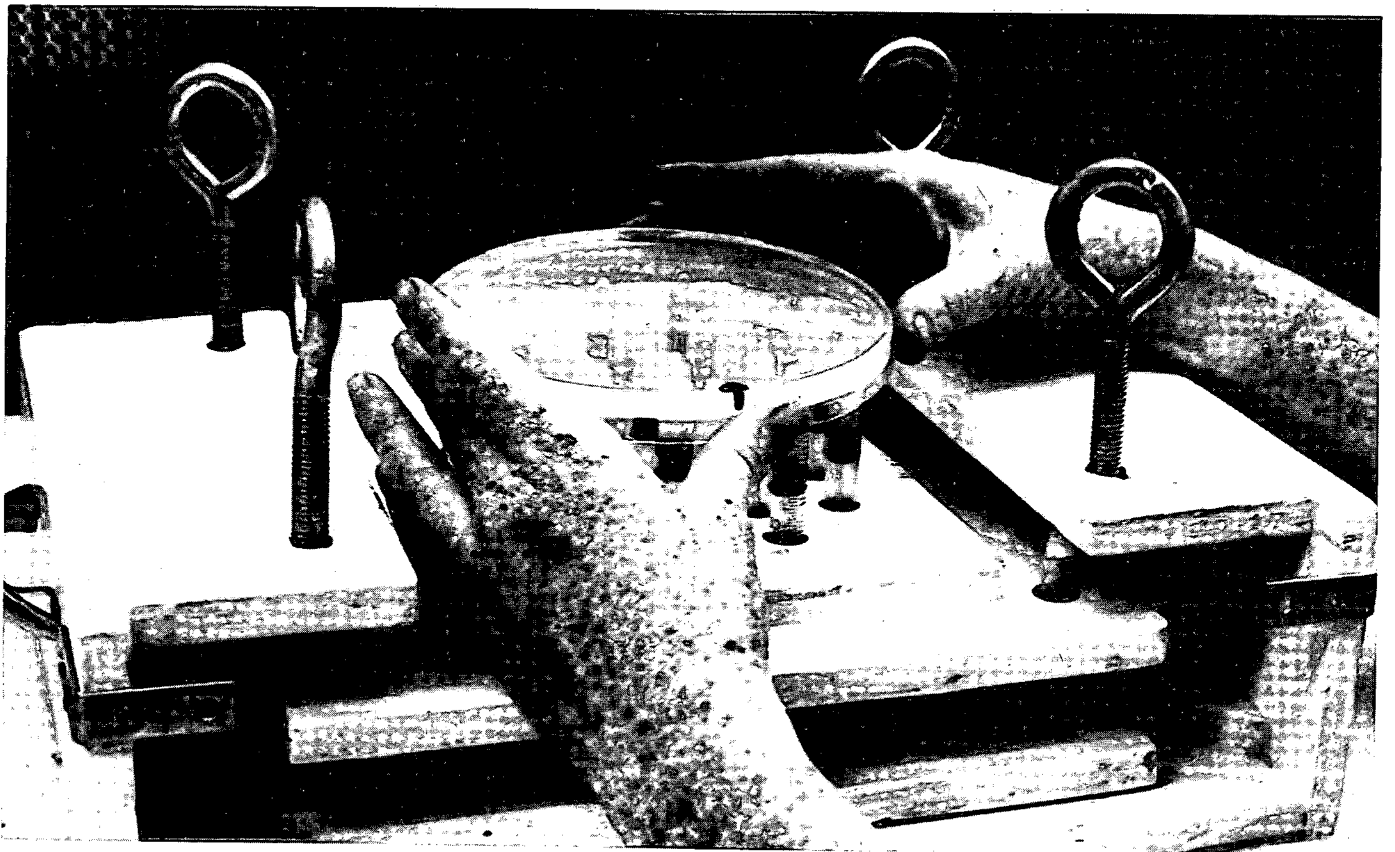


Plate 4. Inoculator for plates with all the syringes in place in preparation for inoculation. During the inoculation process 15 cm diameter agar plates were inoculated by just touching the droplets on the syringes.

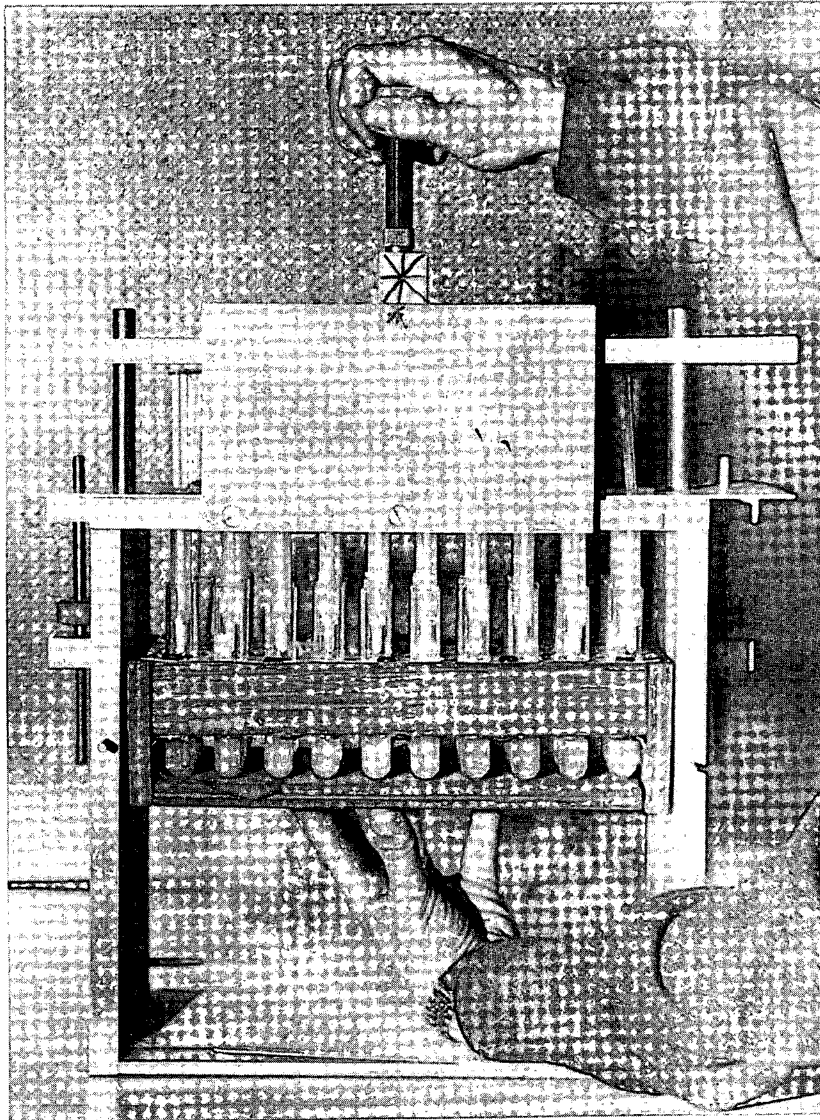


Plate 5. Inoculator for tubes during the inoculation process. A rack containing forty test tubes was inserted and the inoculum was dispensed by turning the top screw.

Numerical Analysis

L. J. Wuest

IBM cards were punched with the test results, using a simple plus minus, binary system. "No test" was also recorded. The code was 1 for positive, 0 for negative and 3 for no test. The NT-Z program using an IBM 370/158 VS-2 computer (unless otherwise specified) was used to analyze the data. NT-Z is a modification of the NT-SYS Program obtained from F. J. Rohlf, State University of New York, Stoney Brook (Sneath, 1957b; Sneath, 1958; Sneath and Cowan, 1958; Sneath and Sokal, 1973).

The analysis as originally programmed by NT-SYS was a three-step process.

The similarity coefficient for each isolate, as compared with the other isolates, was calculated by the Jaccard coefficient as recommended by Sneath.

$$S_J = \frac{a}{a+u} = \frac{\text{Positive Matches}}{\text{Positive Matches + Mismatches}}$$

This association coefficient provided a more accurate reflection of similarity than certain other coefficients, which may either weight positive matches giving them more emphasis or utilize negative matches. Both of the latter systems are risky in bacteriological work.

The characters utilized were all single state; in other words, the result is expressed as positive or negative. Multistate characters often depend on subjective evaluation and increase the possibility of error.

The second stage is the cluster analysis. We have used the single linkage method and/or the unweighted pair group with arithmetic averaging (UPGMA).

Data sets have been prepared for the following analyses:

1. Jaccard coefficient. (S_J)

2. Clustering method. Each coefficient has been clustered by three methods:
   (i) single linkage
   (ii) unweighted pair grouping with arithmetic averaging
   (iii) complete linkage

Each of the data sets contained the following information:

1. Input data - The data for each strain was recorded and printed out by the computer as it appeared on the computer cards.

2. Positive Test Percentage - The positive test percentage for each strain was given along with a separate breakdown of the positive percentage of the substrate utilization tests.

3. Tree Matrix of Cluster Results - This matrix contained a summary of the linking levels of each strain with every other strain or group of strains as they would appear in a tabular dendrogram.

4. Cluster Analysis - Clustering at 75% or greater.

5. Analysis of Feature Frequency Versus Clusters - Each test was examined to determine the percentage of positive organisms within a given cluster for that test.

6. Mean Intergroup Coefficients - The similarity coefficients of the strains of one cluster were averaged with the similarity coefficients of the strains of another cluster yielding a mean similarity coefficient for the two groups.

7. Mean Intragroup Coefficients - This coefficient was obtained by taking the average of the similarity coefficients of the strains within a cluster.

8. Mean Cluster Substrate Utilization - Any given cluster was examined to determine how many, of all the tests, were positive as well as how many of the substrate utilization tests were positive for that cluster.

9. Mean Interstrain Coefficients - In a data set, the similarity coefficient of each strain with everv other strain was averaged out.

10. Cluster Centroids - The similarity coefficients of all strains within a cluster were averaged with each other. The strain with the highest mean or that which was the most similar to all the others was the centroid strain for that cluster.

11. Association Coefficient Matrix - This was a symbolic representation of the clusters found in the tree matrix.

Cluster Identification

Each cluster centroid strain as well as several representatives of the cluster were tested for Gram reaction, cell size and colonial morphology, poly-β-hydroxybutyrate accumulation (Franklin & Franklin, 1971), motility and presence and type of flagellation (Liefson, 1960).

The first region or cluster extended from OTU * SE75W031 to OTU SE75Z087 (Figure 4).

	SE75W031	SE75Z047	SE75Z041	SE75Z055	SE75Z085	SE75Z090	SE75Z087
SE75W031	X						
SE75Z047	.8	X					
SE75Z041	.9	.9	X				
SE75Z055	.9	.9	.95	X			
SE75Z085	.9	.8	.9	.95	X		
SE75Z090	.8	.8	.9	.95	.9	X	
SE75Z087	.75	.75	.9	.9	.9	.9	X

* OTU - operational taxonomic unit.

Figure 4. Region one, similarity coefficients for pairs of OTU's.

Single Linkage versus UPGMA

M.A. Holder-Franklin, C.J. Cormier and L.J. Wuest

Single linkage and UPGMA clustering are two common methods of clustering employed in numerical taxonomy. The most suitable way to compare these two methods of grouping OTU's is to discuss each clustering procedure using a set of data extracted from an association coefficient matrix. Coefficients from the September matrix (Cormier, 1978) were used to obtain this data set. Figure 4 is a triangular matrix showing similarity coefficients for pairs of OTU's. The initial comparison steps have been carried out but are not included in this figure. Shown here are the rearranged OTU's after they have been aligned into groups of highly similar organisms. The OTU's were rearranged into this matrix, leaving two distinct regions observable, with the intragroup similarities of each greater than 0.75.

The second region began at OTU SE75Z066 and ended with the last OTU in the matrix, SE75Z082 (Figure 5).

	SE75Z066	SE75Z074	SE75Z077	SE75Z079	SE75Z082
SE75Z066	X				
SE75Z074	.8	X			
SE75Z077	.9	.9	X		
SE75Z079	.9	.9	.9	X	
SE75Z082	.8	.9	.9	.9	X

Figure 5. Region two, similarity coefficients for pairs of OTU's.

Each of these groups exhibited high intragroup similarity coefficients. Cluster one had an intragroup similarity coefficient value of 0.832. The value in cluster two was 0.832.

In order to get two established clusters to link, each organism featured in the first cluster must be compared to each organism in the other cluster. If at least one comparison exists, where the similarity between two organisms, one in each cluster, is greater than 0.75, then the clusters will become one, via this single linkage.

	SE75W031	SE75Z047	SE75Z041	SE75Z055	SE75Z085	SE75Z090	SE75Z087	SE75Z066	SE75Z074	SE75Z077	SE75Z079	SE75Z082
SE75W031	X											
SE75Z047	.8	X										
SE75Z041	.9	.9	X									
SE75Z055	.9	.9	.95	X								
SE75Z085	.9	.8	.9	.95	X							
SE75Z090	.8	.8	.9	.95	.9	X						
SE75Z087	.75	.75	.9	.9	.9	.9	X					
SE75Z066	.7	.7	.7	.7	.7	.7	.6	X				
SE75Z074	.7	.7	.8	.8	.7	.7	.7	.8	X			
SE75Z077	.7	.8	.9	.8	.7	.7	.7	.9	.9	X		
SE75Z079	.7	.7	.9	.8	.7	.7	.7	.9	.9	.9	X	
SE75Z082	.7	.7	.8	.7	.7	.7	.7	.8	.9	.9	.9	X

Figure 6. A triangular matrix showing similarity coefficients for pairs of OTU's and how they are clustered using the single linkage clustering method.

In Figure 6, eight such links existed.

SE75Z074 ———— .8 ———— SE75Z041
SE75Z074 ———— .8 ———— SE75Z055
SE75Z077 ———— .8 ———— SE75Z047
SE75Z077 ———— .9 ———— SE75Z041
SE75Z077 ———— .8 ———— SE75Z055
SE75Z079 ———— .9 ———— SE75Z041
SE75Z079 ———— .8 ———— SE75Z055
SE75Z082 ———— .8 ———— SE75Z041

The final cluster that would appear for the OTU's SE75W031 to SE75Z082 as clustered by single linkage clustering is shown in Figure 6. One single cluster has formed with a lower intragroup similarity value. This illustrates the loose, low similarity clustering characteristic of the single linkage method.

UPGMA Clustering

The criteria involved for UPGMA clustering are considerably more stringent. Again, before an attempt is made to link two established clusters, each organism in one cluster must be compared to every other organism in the other cluster. The total arithmetic average is determined by adding up the similarity values obtained for each comparison made between each pair of organisms, then dividing this sum by the number of values.

The general formula for determining the average intragroup similarity of a group of n strains is given by

$$\frac{\sum_{j=2}^{n} \sum_{i=1}^{j-1} S_{ij}}{\frac{n(n-1)}{2}}$$

where S_{ij} is the coefficient of association between strain i and strain j and n is the number of strains.

	SE75W031	SE75Z047	SE75Z041	SE75Z055	SE75Z085	SE75Z090	SE75Z087	SE75Z066	SE75Z074	SE75Z077	SE75Z079	SE75Z082
SE75W031	X											
SE75Z047	.8	X										
SE75Z041	.9	.9	X									
SE75Z055	.9	.9	.95	X								
SE75Z085	.9	.8	.9	.95	X							
SE75Z090	.8	.8	.9	.95	.9	X						
SE75Z087	.75	.75	.9	.9	.9	.9	X					
SE75Z066	.7	.7	.7	.7	.7	.7	.6	X				
SE75Z074	.7	.7	.8	.8	.7	.7	.7	.8	X			
SE75Z077	.7	.8	.9	.8	.7	.7	.7	.9	.9	X		
SE75Z079	.7	.7	.9	.8	.7	.7	.7	.9	.9	.9	X	
SE75Z082	.7	.7	.8	.7	.7	.7	.7	.8	.9	.9	.9	X

Figure 7. A triangular matrix showing similarity coefficients for pairs of OTU's and how they are clustered using UPGMA clustering method.

In this data set, Figure 7, 1/35 (25.4) = 0.72, i.e. the intergroup similarity existing between these clusters is 0.72. Since the mean similarity value is below the 0.75 cutoff point, the two clusters will not link, but will remain as they are in Figure 7. Rather than linking and becoming a cluster of lower overall similarity, each cluster remains tightly packed, hyperspherical and discrete, characteristic of UPGMA clustering.

September Single Linkage Clustering and UPGMA Clustering

A comparison of the two clustering methods, as shown in Figures 6 and 7 for the September sampling period, demonstrated that major differences were observed involving the size of the clusters formed, i.e. in the number of isolates incorporated per cluster as well as the levels of the mean intragroup similarity coefficients. Single linkage clusters were larger and contained low intragroup similarities. The reverse was true for UPGMA. Clusters in the averaging method were smaller with high intragroup mean similarity coefficient values.

Single Linkage, UPGMA and Complete Linkage Clustering Compared

The various methods provide major clusters which are very similar, using reference strains. However, the UPGMA appears to provide groupings which are the closest to the taxa previously described by Stanier, Doudoroff & Palleroni (1966). Table 2 contains a comparison of the three methods using reference strains only. The clusters selected by the computer using UPGMA and CL re identical with the Pseudomonas taxospecies design ted by Stanier. The single linkage technique did not contain this refinement.

According to Stanier et al. P. chlororaphis could be considered to be Pseudomonas fluorescens biotype D, and P. aureofaciens, P. fluorescens, biotype E. These strains are similar enough to appear in the same cluster.

The association coefficient matrix produced by the UPGMA method was in good agreement with the clustering results and the tree matrix. The CL results contained more discrepancies, such as the ordering of the reference strains. Pseudomonas marginata nd Pseudomonas cepacia were in the seventh and eighth positions in the association coefficient matrix nd showed a 70-75% similarity, which was in agreement with the tree matrix appearing at the 0.724 level. However, they do not appear in a similar position in the clusters selected by the computer. Taxonomically, these strains are associated with each other but do not belong in the taxa designated in Table 2.

In general, UPGMA clustering provided the most compact and discrete of the three clustering methods.

DNA analysis

A rapid method for the determination of the % G + C content of DNA was developed for the taxonomy study (Cashion et al. 1977).

Table 2. Clusters selected by computer.

Data shown September, Reference Strains, S_J

Comparison of:
1. Single Linkage (SL)
2. Unweighted Pair Group Arithmetic Averaging Clustering (UPGMA)
3. Complete Linkage (CL)

Clustering Technique			Strain
UPGMA	CL	SL	
Cluster 1	1	1	181 Pseudomonas chlororaphis ATCC 9446-a
			255 Pseudomonas chlororaphis ATCC 9446-b
			215 Pseudomonas aureofaciens ATTC 13985
Cluster 2	2	2	182 Pseudomonas fluorescens ATCC 13525-a
			205 Pseudomonas fluorescens LCDC
			214 Pseudomonas fluorescens ATCC 13525-b
Cluster 3	3	3	183 Pseudomonas aeruginosa LCDC
			198 Pseudomonas aeruginosa ATCC 15442
			212 Pseudomonas aeruginosa ATCC 14216
Cluster 4	4	4	191 Pseudomonas putida LCDC
			213 Pseudomonas putida ATCC 12633

LCDC -- Laboratory Centre for Disease Control (Ottawa)
ATCC -- American Type Culture Collection (Washington)

An Introductory Study of Seasonal Variation in the Aerobic Heterotrophic Bacterial Population of the Saint John River Using Classical Systematics

M.J. Chalifour and M.A. Holder-Franklin

SUMMARY

Subsurface water samples were collected from the Saint John River in October, February and August, one mile above and one mile below the effluent outflows of a food processing plant and a dairy. Aerobic heterotrophic bacteria were isolated, enumerated and identified where possible. The isolates were also examined for enzyme activity. The temperature and pH of the river water were determined at the time of sampling and the 24 hour mean flow rate between sampling locations was recorded. The fluctuation in the genera of bacteria observed indicated that seasonal factors influenced the bacterial population. Many of the isolates required growth factors; the most common one was cyanocobalamin. The ability to store poly-beta-hydroxybutyrate was prevalent in the summer isolates, rare in the winter bacteria.

The predominant bacteria in the winter samples were Alcaligenes, Flavobacterium, fluorescent Pseudomonas and Xanthomonas species. Zoogloea species were dominant in August. Chromobacterium, Enterobacteriaceae and non-fluorescent Pseudomonas, as well as Zoogloea, were predominant in the October samples.

INTRODUCTION

The importance of heterotrophic bacteria in the biological self-purification of rivers and streams is well known and has been stressed in reviews by Wurhmann (1972) and Velz (1970). The seasonal variation of these bacteria in estuarine waters has been described by Sieburth (1967) and the distribution of such bacteria found in fresh water lakes has been well documented by Collins (1963).

The initial purpose in the identification of the bacterial species of the river was to determine the predominant heterotrophic species at the various seasons. The preliminary study reported here was designed to determine if a population shift could be detected and, if possible, the nature of the shift in the subsurface water samples.

The effluent entering the river midpoint between the sampling stations was from three major sources: the washings from potato processing which did have primary treatment to remove sedimentable wastes, the untreated human sewage from this plant, and the untreated washings from a small dairy. Several small streams draining tilled land entered at various points along the river. The river front community has a low population density. When the plant was in full operation, however, the human waste effluent was increased.

Because of the nature of the effluent, several biological parameters were studied.

The extracellular enzymes investigated were amylase, pectinase, caseinase and phosphatase. General population changes were indicated by the presence or absence of fluorescence and the ability to grow on MacConkey agar.

MATERIALS AND METHODS

Sources and Treatment of Samples

River water samples were collected between 1:00 p.m. and 3:00 p.m. in October 1970, February and August 1971. The sampling points were two miles apart, above and below the village of Florenceville, encompassing a section of river subjected to a variety of domestic, agricultural and food processing wastes. The upstream and downstream locations were designated the upper and lower sampling stations. The average rate of flow recorded at the Provincial Recording Station situated at the same location was $54.7 \times 10^6 cm^3.s$ (October sample), $16.5 \times 10^6 cm^3.s$ (February), and $64.0 \times 10^6 cm^3.s$ (August). The river is 500 m wide at the sampling sites.

Sampling Procedure

The summer and autumn samples were collected at three points at each sampling station. The first point was 30 m from the east bank, the second at mid-river, and the third, 30 m from the west bank. In October and August, six samples were obtained. The winter samples were collected at mid-river only, following the removal of a 60-cm thickness of ice by drilling holes 20 cm in diameter with an ice borer.

All samples were collected in sterile 1000-ml nalgene screw-cap bottles by facing the opened bottles upstream and plunging them just below the surface of the water. The samples were placed in crushed ice for transportation to the laboratory. The pH and temperature of the river water were recorded at the time of sampling.

Isolation and Enumeration of Colonies

Serial dilutions of the samples were made in distilled water. From each dilution, 0.1 and 0.2 ml were surface-plated on the casein peptone starch (CPS) medium of Collins & Willoughby (1962). MacConkey agar plates were inoculated with 0.2 ml of each dilution on the surface. All plates were prepared in triplicate and incubated for 72 hours at 20°C, except for the CPS plates made from the mid-winter samples which were incubated

Table 3. Determinative Tests for Classification of Gram-Negative Rods

Characteristics	Acinetobacter	Aeromonas	Alcaligenes	Chromobacterium lividum	Enterobacteriaceae	Flavobacterium	Moraxella	Pseudomonas	Xanthomonas	Zoogloea	Cellvibrio
Flagella	-	P	Pe	P	Pe	Pe	-	P	P	P	P
Pigment	-	-	-	V	-	C	-	F	C	S	-
pbhb	-	-	+	+	-	-	+	v	-	+	-
Growth Factors	-	-	B-12	-	v	+	+	v	+	B-12	+
Enzymes											
Amylase	-	+	-	-	v	+	-	v	+	-	-
Arginine dihydrolase	-	+	-	-	-	-	-	v	-	-	-
Caseinase	-	+	-	-	v	v	-	v	+	-	-
Catalase	w	+	+	+	+	w	-	+	+	w	+
Oxidase	-	+	+	+	+	w	+	+	+	w	+
Phosphatase	-	-	-	-	-	v	-	v	+	-	-
Pectinase	-	-	-	-	v	-	-	v	v	-	-
O.F. Glucose	-	+	-	+	+	-	-	v	+	-	-
O.F. Xylose	+	+	-	+	v	-	-	v	+	-	-
O.F. Lactose	-	+	-	+	v	-	-	-	+	-	-
**Acetate	+	+	+	+	+	-	+	+	+	+	-
Cellulose	-	-	-	-	-	-	-	-	-	-	+
p-Hydroxy-benzoate	+	-	v	+	w	-	-	+	-	+	-
Aromatic ring cleavage	o		m	-*	-	-	-	v		m	
Growth in NO_3	-	+	+	+	+	-	-	v	-	-	-

P - polar; Pe - peritrichous; V - violacein; C - carotenoid; F - fluorescent;
S - straw coloured; pbhb - poly-beta-hydroxybutyrate storage; v - variable;
B-12 - cobalamin,; o - ortho; m - meta.
* - pigment interfered with reaction; ** carbon sources utilized.
O - oxidative; F - fermentative.

Principal References for taxonomy

Acinetobacter: - Baumann et al. 1968: Baumann, 1969: Canovas & Stanier, 1967: Henrikson & Bovre, 1968.
Aeromonas: - Eddy, 1960: Eddy, 1962.
Alcaligenes: - Board, 1965: Burdon, 1946: Thornley, 1968.
Chromobacterium: - Sneath, 1966.
Enterobacteriaceae: - Stanier et al. 1970.
Flavobacterium: - Hendri et al. 1968.
Moraxella: - Baumann et al. 1968.
Pseudomonas: - Palleroni & Doudoroff, 1972: Stanier et al. 1966.
Xanthomonas: - Hayward & Hodgekiss, 1961.
Zoogloea: - Crabtree et al. 1965: Freidman & Dugan, 1968. Unz & Dondero, 1967.
Cellvibrio: - Stanier et al. 1966.

for 4 days at 5°C. Culture plates which contained between 80 and 150 colonies were used to determine the number of viable bacteria per millilitre.

A total coliform count was made on the samples of river-water collected in the summer season. A membrane filter technique using Difco-M-Endo Broth was used (Standard methods for the examination of water and waste water, 13th ed., American Public Health Association, Washington, D.C.). The culture plates were placed in cans with dampened filter paper to maintain a moist atmosphere and incubated 24 hours at 35°C. The membrane filters were dried at room temperature for one hour. The number of colonies with a golden green metallic surface lustre were interpreted as being members of the coliform group of bacteria and were recorded as coliforms per 100 ml.

Isolation and Maintenance of Cultures

One hundred colonies were selected randomly from each seasonal sample and further purified on CPS plates. Fifty colonies were selected from samples obtained from above the effluent outflow and fifty colonies from below. The isolates were carried on CPS plates and replated each month. Reference cultures were maintained in screw-cap tubes of CPS semi-solid agar at 4°C and subcultured every six months.

In order to estimate the number of coliform type organisms appearing in the river, 50 colonies from each seasonal sample, 25 from each sampling station, were randomly selected from MacConkey plates and stabbed and streaked onto slopes of Bacto-Triple Sugar Iron agar, at pH 7.4, incubated from 72 hours at 20°C and examined for the production of acid and hydrogen sulfide.

Identification Methods

The organisms were identified according to genera using the determinative method. A summary of the generic descriptions is given in Table 3. Determinative tests, unless otherwise stated, were carried out on cultures incubated at 20°C for 48 hours.

Gram Stain

Colonies of each isolate were emulsified in sterile distilled water on a slide, air dried, heat fixed and Gram stained (Blair et al. 1970).

Colonial Morphology

Well-isolated colonies were examined for colonial shape, size, colour and texture by use of a stereomicroscope at 10x magnification after growth on CPS medium.

Oxidation or Fermentation of Carbohydrates (0-F test)

The O-F test was determined for glucose, lactose and xylose by the method of Hugh & Leifson (1953).

Fluorescent Pigments

Growth on medium B of King et al. (1954), as well as on nutrient milk agar, was examined for fluorescence using ultraviolet light at 360 mμ.

Phenazine Pigments

Growth on medium A of King et al. (1954) was examined for diffusible pigment after 7 days.

Carotenoid Pigments

Growth on CPS and nutrient agar was examined after 7 days for nondiffusible yellow pigment.

Violacien Pigments

Purple pigmented bacteria growing on CPS medium were tested for violacien according to the method of Sneath (1966).

Flagella

Organisms cultured in CPS broth were stained by the Leifson (1960) method (modification Cornell et al. 1968). Many isolates were difficult to stain by this method, because of the presence of slime or of capsular matrices. In these cases negatively stained preparations made according to the method of Baillie et al. (1962) were examined by means of a Phillips EM electron microscope.

Motility

1) Hanging drop method: 1 ml of a 48 hours CPS broth culture was transferred to 10 ml of fresh broth at 20°C and incubated a further 6 hours before examination by phase contrast microscopy.

2) Spreading growth on the surface of CPS semi-solid medium from a small central inoculum was observed.

3) Electron microscopy (Phillips EM 200) was used on organisms which presented conflicting results by the two previous methods.

Growth Factor Requirements

The auxanographic technique described bv Pontecarvo (1949) was utilized to determine required growth factors. The underlayer of full strength agar consisted of the mineral salts medium of Stanier et al. (1966) with 1% (w/v) sodium acetate, sodium succinate and DL-lactate as carbon sources. A soft ion agar (0.7%) overlay was inoculated with bacteria which had been previously washed free of growth medium. Growth factors were then spotted on the surface in the following combinations.

1) 0.1% vitamin-free casein plus 10 μg/ml tryptophan as a source of amino acids.

2) 0.3% yeast extract, which supplies a variety of factors including purines, pyrimidines, amino acids, peptides and vitamins.

3) 10 g/ml methionine.

4) a mixture of the pantothenate (1 μg + ml), biotin (0.005 μg/ml) and cyanocobalamin (1 μg/ml) plus 10 g/ml of cystine.

5) a mixture of pantothenate (1 μg/ml), biotin (0.005 g/ml) and cyanocobalamin (1 μg/ml).

6) a mixture of biotin (0.005 μg/ml) and cyanocobalamin (1 μg/ml).

7) biotin (0.005 μg/ml)

8) cyanocobalamin (1 μg/ml)

Poly-Beta Hydroxybutyrate Storage

Smears were prepared from colonies grown for 48 hours on mineral-salts medium with 0.1% sodium acetate as the carbon source. The smears were allowed to air dry and were stained by the method of Burdon (1946) for the presence of lipid material. Those isolates which were unable to grow on this medium were selected from plates supplemented with the required growth factors.

Catalase Activity

Drops of 10% hydrogen peroxide were added to 24 hour yeast extract agar cultures according to the method of Rhodes (1959) and examined for gas bubbles.

Oxidase Reaction

Klinge's method (1960), modified by adding a few drops of a freshly prepared 1% (w/v) aqueous solution of p-aminodimethylaniline oxalate to CPS plate cultures and examining colonies after 30 seconds for a purple colouration, was used.

Phosphatase

The method of Barber & Kuper (1951) was modified by adding 0.1% sodium phenolphthalein phosphate to CPS medium. Spot cultures replicated and incubated on this medium were exposed to ammonium hydroxide to develop the presence of free phenolphthalein.

Caseinase

Spot cultures replicated on CPS medium were flooded with 30% trichloracetic acid according to the method of Pitt & Dey (1970). The presence of clear zones around colonies indicated the production of caseinase.

Amylase

Spot cultures replicated on CPS medium were flooded with Gram's iodine; clear and reddish zones indicated hydrolysis (Manual of Microbiological Methods, 1970).

Pectinase

Spot cultures replicated on the polypectate gel medium of Hildebrand (1971) at a pH of 5.0 and 8.0 were examined after 72 hours at 20°C for pit formation.

Utilization of Carbon Compounds

The replica plating method of Lederberg & Lederberg (1952) was used for the determination of the utilization of acetate, cellulose, ethanol and para-hydroxybenzoate. Replica plates were prepared on the basal medium of Stanier et al. (1966) and on the same medium with addition of the appropriate carbon source. The plates were incubated at 20°C for 7 days and isolates were presumed to utilize the carbon source if growth increased.

Aromatic Ring Cleavage

Strains capable of growth on the minimal medium of Stanier et al. 1966, containing para-hydroxybenzoate, were tested for ortho or meta cleavage of protocatechuate (Hugh, 1970).

Potato Rotting Ability

Fresh disease-free potatoes were washed, peeled, disinfected in 10% sodium hypochlorite, cut into 1-cm squares, and heavily inoculated with bacteria. After incubation at 22°C for three days, the potato surface was examined for soft rot (Lelliot et al. 1966).

Arginine Dihydrolase Activity

The presence of a constitutive arginine dihydrolase system was determined (Thornley, 1960).

Nitrogen Sources for Growth

All strains were tested for their ability to use ammonium and nitrate salts as a nitrogen source and to fix atmospheric nitrogen. Tests were carried out in the mineral base broth of Stanier et al. (1966) with either $(NH_4)_2SO_4$ or KNO_3 (1 g/l) or without a nitrogen source. The carbon source used was a mixture of acetate, lactate and succinate, each at 0.1% (w/v). Tubes containing 5 ml of broth were inoculated with organisms taken from CPS agar cultures. The tubes were placed in a slanted position to allow maximum absorption of atmospheric gases and incubated at 20°C for 72 hours.

RESULTS

Distribution of Bacterial Populations

The viable plate counts on CPS and MacConkey medium, together with the reactions produced by isolates randomly selected from the MacConkey plates on Triple Sugar Iron agar slopes, are compared in Table 4. The viable counts shown as CFU/ml on CPS medium were similar at all sampling stations and seasons. The bacterial counts in samples obtained in October and August at three stations across the river were similar as were the counts recorded from the upper and lower sampling stations. The final counts represent the average of the counts from all sampling stations. The strains isolated were representative of the total population and selected randomly from the plates. All of the organisms isolated from the CPS medium were gram-negative rod-shaped bacteria without sheaths or prosthecae. However, a seasonal population shift had occurred without a significant change in total numbers. A distinctive difference was observed seasonally in the numbers of organisms isolated on MacConkey agar, which is a selective medium. MacConkey agar supports the growth of the coliforms and other fecal gram-negative rods as well as many of the Pseudomonas group, however, many

Table 4. Temperature and pH of River Water; Viable Counts (CFU/ml) and Fermentation Reactions of Bacteria from River Water Samples.

Date	Temperature °C	Location	pH	COUNTS (CFU/ml) Collin's Agar	MacConkey's	FERMENTATION Glucose	Glucose Lactose and Saccharose
Oct.71	9	Above	7.3	3×10^5	5×10^4	6%	24%
	9	Below	7.3	2×10^5	3×10^4	12%	10%
Feb.72	0	Above	7.2	1×10^5	8×10^4	0	0
	0	Below	7.0	9×10^4	8×10^4	0	0
Aug.72	20	Above	7.3	7×10^4	2×10^2	11%	55%
	20	Below	7.3	3×10^4	4×10^2	0	68%

species are suppressed on MacConkey. The increase in growth on the MacConkey medium in February with the concomitant disappearance of the fermentative groups as indicated by the negative tests on the triple sugar iron agar clearly follows the appearance of a greater number of Pseudomonas. This was substantiated by the results of the determinative tests.

The decrease in numbers on MacConkey in August also follows the drop in the Pseudomonas group. The majority of the organisms observed in the August samples were fermenters as shown by the T.S.I. test (Figure 8).

August samples (Figure 9)

Zoogloea species predominated in August (63% and 78% above and below Florenceville respectively). Non-fluorescent Pseudomonas and Xanthomonas species were common to both sampling areas, while Acinetobacter and Aeromonas types were observed only at the upper station. Isolates (2%) from the lower station were identified as Enterobacteriaceae. The method was designed to select the predominant organisms, therefore, a shift in numbers below the detectable level of the method could add or remove those organisms which had not clearly established supremacy in the population.

February Samples (Figure 9)

The genus Pseudomonas, particularly the fluorescent species, was dominant among the February isolates. Forty five percent of the isolates from the upper station and 83% from the lower station belonged to this genus; 54% of the former and 94% of the latter were fluorescent species. Xanthomonas and Flavobacterium species were observed in the upper station samples. Alcaligenes species were found from both sampling areas.

October Samples (Figure 9)

A greater diversity in bacterial population was apparent in the October samples from which 10 genera were isolated. Over 50% of the isolates from both sampling areas belonged to the genus Pseudomonas.

Enterobacteriaceae and Zoogloea species were prevalent in samples from both areas, each comprising about 10% of the population. Alcaligenes, Cellvibrio, Chromabacterium, Moraxella, Xanthomonas and Flavobacterium were also observed.

Bacterial Metabolic Activities

The characteristic activities of the bacterial isolates are presented graphically in Figure 10.

The production of extracellular enzymes was more pronounced in the bacterial isolates from the October and February samples than in those from August.

Amylase production was characteristic of 36% and 50% of the isolates from the upper station in October and February and in 42% of the isolates from the lower station in October. However, the August samples contained only 10% and 2% amylase producers, from the upper and lower stations respectively. These amylolytic bacteria were non-fluorescent Pseudomonas, Enterobacteriaceae, Flavobacterium and Xanthomonas species. Few amylolytic isolates were found from the lower station in February, reflecting the predominance of fluorescent Pseudomonas species in those samples.

Pectinase

The samples from October contained a high percentage of pectolytic organisms, 26% of which were isolated from the upper station and 18% from the lower station. Four percent of the isolates from February and August were pectolytic. This activity was carried out by non-fluorescent Pseudomonas and Xanthomonas species and species of Enterobacteriaceae.

Phosphatase

The ability to produce phosphatase was found in 30% of the organisms isolated in October and from the

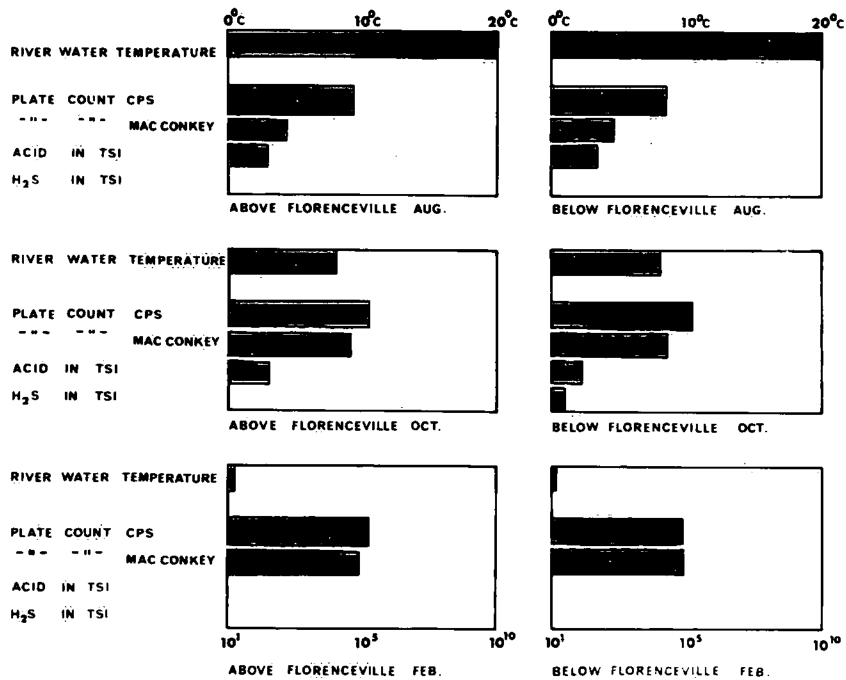


Figure 8.

Comparisons of river water temperature and counts on casein peptone starch (CPS) and MacConkey agars. Acid reaction and H_2S production in triple sugar iron agar (T.S.I.) in six samples.

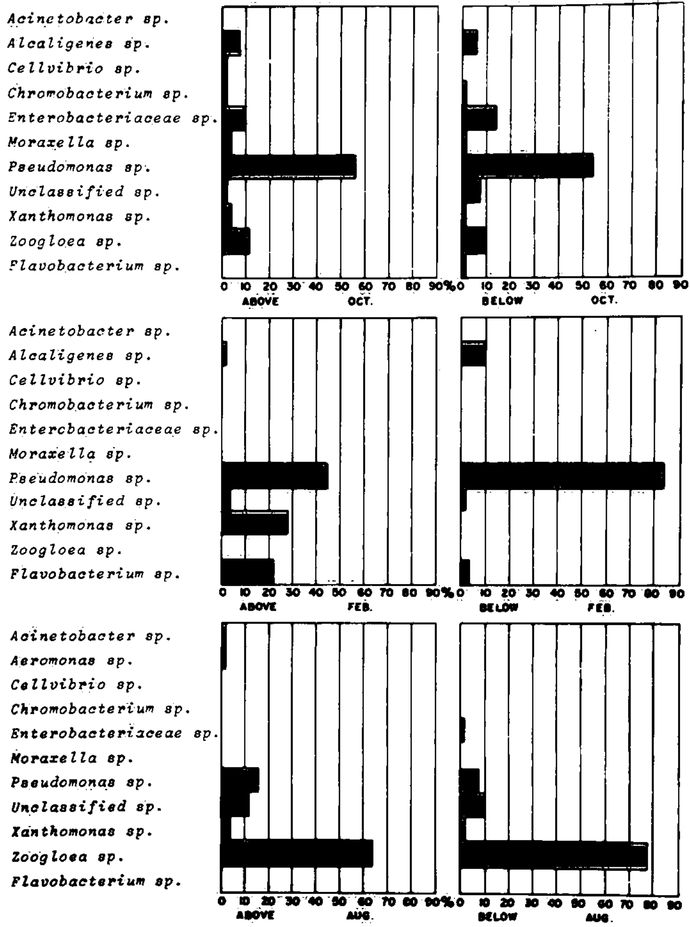


Figure 9. Distribution of bacterial genera in the various samples.

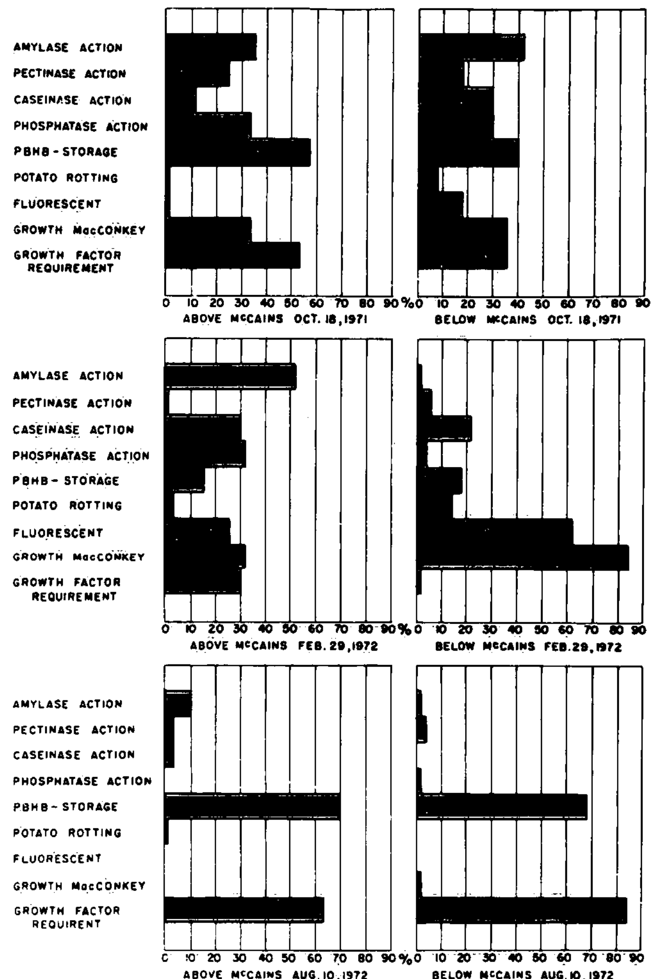


Figure 10. Certain characteristics of the organisms isolated from the various samples.

upper station in February. Only 2% of the isolates in August and from below the village in February were phosphatase producing and included non-fluorescent Pseudomonas, Xanthomonas and Enterobacteriaceae species.

Caseinase

The ability to produce caseinase was demonstrated in over 25% of the February and 20% of the October isolates and in only 4% of the August bacteria, and included fluorescent and non-fluorescent Pseudomonas species, Aeromonas, Flavobacterium, Xanthomonas and Enterobacteriaceae species.

Poly-beta-hydroxybutyrate storage was found in 70% of the August, 50% of the October and less than 20% of the February isolates. The organisms storing poly-beta-hydroxybutyrate were Alcaligenes, Chromobacterium, non-fluorescent Pseudomonas and Zoogloea species.

Growth on MacConkey

Growth in the presence of bile salts on MacConkey medium was a characteristic quality of over 80% of the isolates from above and 30% from below the village in February, and in over 30% of those isolated in October.

Growth Factor Requirement

It was found that 60% and 80% of the isolates collected in August, 54% and 36% in October and 30% and 2% in February were unable to grow in the described mineral salts broth, required extra growth factors and included species belonging to the genus Flavobacterium, Moraxella, Pseudomonas, Xanthomonas and Zoogloea and to the family Enterobacteriaceae.

The screening test was performed on all isolates requiring growth factors. Cyanocobalamin was essential for the growth of 77% of the bacteria isolated in August, 15% of the October isolates and 1% of the bacteria isolated in February. These bacteria belonged to the genus Zoogloea except for a single February isolate which was classified in the genus Alcaligenes.

Biotin was required by three Pseudomonas species, and a mixture of biotin, cyanocobalamin, cystine and pantothenate was required by a single Pseudomonas isolate.

Growth factors not determined by the screening methods were required by 24 isolates from the October, 22 from the February and 11 from the August samples. These isolates were mainly Flavobacterium or Xanthomonas species.

The Most Favourable Temperature for Growth

An estimate of the optimum temperature for colony growth of the isolates was made by measuring colony size at 37°C, 20°C and 4°C - 6°C and is presented graphically in Fig. 11. This method was based on one used by Johnson et al. (1970) in which they were able to determine the specific growth rate of Pseudomonas fluorescens by measuring colony size on a solid medium. Ninety-eight percent of the isolates from the February samples grew best at 20°C, whereas 56% of the October isolates grew best at 20°C, 35% at 37°C and 5% at 6°C.

Table 5. Viable count of coliforms (CFU/100 ml), August, 1972

Location	Coliforms /100 ml
Above, East bank	1×10^3
Midstream	1×10^3
West bank	7×10^3
Below, East bank	2×10^3
Midstream	2×10^3
West bank	9×10^2

Coliform Counts in August (Table 5)

The coliform counts at various locations demonstrate a consistency which substantiates the method as well as showing clearly that the effluent from McCains was not contributing to a major increase in the coliform count of the river. The numbers are in excess of the water quality standard for recreational water.

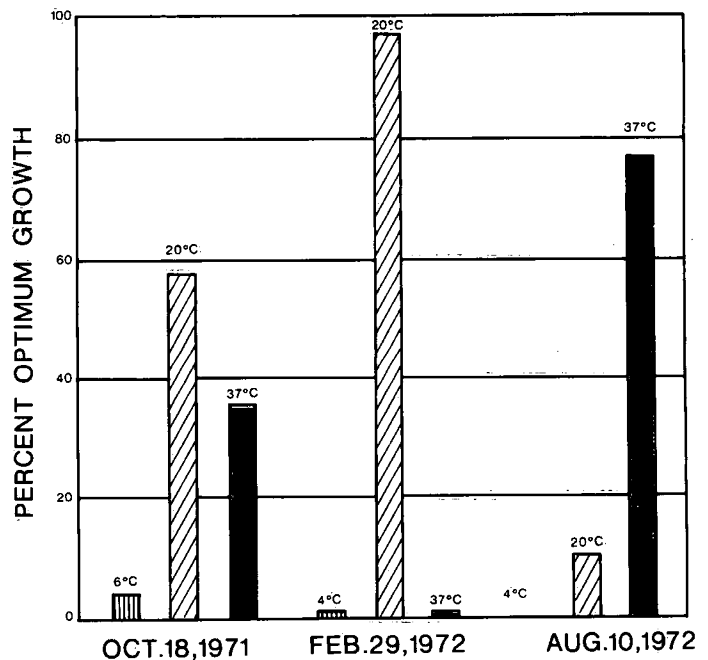


Figure 11. The temperature for optimum growth shown as the percentage of the total population isolated at each of the three seasons.

DISCUSSION

A distinctive difference was observed in the genera isolated in August, October and February. As a

result of the random sampling procedure employed on the dilution plates, the isolates represented the predominant species of bacteria. Species present in low numbers would not be observed. The seasonal differences in species were observed using many criteria, growth on selective media, metabolic activities and identification of certain genera. None of the February isolates were fermentative, but 65% of the bacteria in the August sample catabolized sugars by the fermentative process.

The isolates from the August samples were predominately Zoogloea species, mesophilic organisms unable to grow below 9°C or on MacConkey medium. The October samples contained a heterogeneous population of mesophilic and psychrophilic species. The isolates in the February samples were composed entirely of strictly aerobic facultative psychrophilic bacterial species.

It has been reported by Christopherson (1967) and by Lynch, McLeod & Franklin (1975) that the selection of metabolic pathways occurs as the result of a change in temperature. Christopherson observed that anaerobic pathways are selected at higher temperatures.

According to Velz (1970), the concentration of dissolved oxygen is considerably higher in the winter. The same observation has been made for the Saint John River. The Department of Environment Water Quality monitors have recorded substantial increases in dissolved oxygen with a decrease in temperature.

When these microbial responses and oxygen levels were related to the seasonal isolates in the Saint John River, it seemed probable that the strictly aerobic psychrophiles such as Alcaligenes, fluorescent Pseudomonas and Flavobacterium species would have a selective advantage in the colder months and that the Zoogloea species, which have according to Unz & Dondero (1967) a preference for microaerophilic growth, and the fermentative Enterobacteria would have the advantage in the warmer months.

The pollutants in this area of the river are primarily added nutrients from food processing wastes and human and animal sewage, as well as runoff from heavily fertilized land. Bacterial populations changed in relation to changes in the environment. Two major parameters, temperature and locality, were selected for comparison with the bacteria. It has been shown by the work reported here that populations change in response to temperature. The comparison of the effects of added nutrients from the food processing plants and human sewage of the small community did not reveal a significant change in the bacterial flora.

The ability of the bacteria isolated to degrade organic nutrient wastes was examined seasonally. The predominant organisms in August, Zoogloea species, were distinguished by the production of gelatinous matrices and long flagella. These qualities have been reported by Friedman & Dugan (1968) to induce formation of zoogloeal flocs which have a high binding capacity for metal ions, amino acids and radionuclides. According to Velz (1970), bacterial flocs dispersed throughout a flowing stream are instrumental in biological extraction and transportation of compounds from the water mass into deposits. The ability of Zoogloea species to store poly-beta-hydroxybutyrate and polyphosphate granules would lead, as well, to transportation of microbial reaction products into the sediments. Joyce & Dugan (1970) regarded Zoogloea species in mixed culture as bacterial buffers because they form ketones and esters from organic acids produced by the microbial breakdown of proteins and carbohydrates.

The total coliform count, performed on the August samples, indicated that members of the coli - aerogenes group of bacteria were prevalent at this time. The fermentative ability of these organisms undoubtedly involved them directly in the process of mineralization. The ability of members of this group to produce the vitamin biotin (Filippi & Vennes, 1971) is of even greater significance, as it is stimulatory to the floc-forming Zoogloea (Crabtree et al. 1965).

The February samples contained bacteria whose combined nutritional activities appeared to be complementary in the chain of events leading to the oxidation of reduced organic pollutants. The Xanthomonas and Flavobacterium species isolated were active producers of amylase and phosphatase, while the fluorescent Pseudomonas species were proteolytic and able to utilize carbohydrates. The Alcaligenes species, which were non-glycolytic, utilized acetate, stored poly-beta-hydroxybutyrate and were active ammonifiers.

The organisms isolated in October contained representatives of species found in both the August and February samples as well as several additional ones. A greater variety of Pseudomonas species were present and included, in addition to the fluorescent species, a versatile group which stored poly-beta-hydroxybutyrate and displayed amylolytic activity. Species belonging to the Enterobacteriaceae were among the predominant isolates in October.

More of the bacteria isolated at the colder temperatures produced amylase, pectinase, caseinase and phosphatase - extracellular enzymes capable of initiating the degradation of large organic polymers. These organisms were also more versatile metabolically, were less nutritionally fastidious, and had the ability to adapt to environmental stress.

The population shifts which were most apparent were the decrease in Pseudomonas species in August, and the disappearance of the Enterobacteriaceae and Zoogloea in February.

The nutrients added to the river have some seasonal variation, but there is a continuous source of industrial, domestic and animal sewage. The major population shift was undoubtedly due to the temperature and temperature-related parameters such as dissolved oxygen, while the effluent from the food processing plant did not significantly affect the aerobic heterotrophic bacteria from the river. One of the most significant findings of this research for future studies was the absolute requirement for a variety of vitamins and other growth factors in the predominant bacteria in the summer samples.

An Ecological Approach to the Study of Bacteria at the Florenceville Site in September and February

T. Kaneko, M.A. Holder-Franklin and M. Franklin

SUMMARY

Seasonal river water samples obtained at the point source of the effluent from the McCain food processing plant have been analyzed for numbers and types of heterotrophic bacteria by numerical taxonomy and for temperature and pH. Bacterial counts varied from 10^4 to 10^6 (CFU/ml). Forty per cent of the September river samples required growth factors and 17-32% required unknown growth factors. This growth factor requirement was shown again to be seasonal.

The February isolates were more versatile nutritionally and more of the isolates utilized a wide range of substrates. The winter organisms were able to grow well at a wide temperature range in contrast with the September isolates which did not grow at 4°C.

The starch discharged by the food processing plant did not produce an increase in starch hvdrolyzing bacteria. The samples obtained above and below the point source of the effluent yielded isolates of similar characteristics, whereas the effluent isolates were distinctively different indicating that the effluent bacteria were not retained as a major segment of the river bacterial population. It was concluded that the effluent bacteria were diluted by the river to numbers below detection by the methods employed in this study.

INTRODUCTION

The impact of industrial and domestic wastes on receiving waters is quite apparent when eutrophication, offensive odours or fish-kill are present. However, the ecosystem has already been severely damaged when these phenomena are observed. Therefore, an indicator system is required which provides an early warning that restraint must be employed on the release of toxic or nutrient effluents into water bodies. Conventional methods such as B.O.D. or C.O.D. provide indirect information and very little is known of the relationship of these measurements to biological activity except at the extremes of oxygen levels. There are two questions -- how do the microorganisms degrade pollutants in the natural environment, and what are the environmental factors which suppress or encourage this activity?

In order to investigate the efficacy of a microbial early warning indicator system, we initiated an overall study on the effect of the nutrient effluent of McCain's food processing plant on the bacterial flora of the Saint John River at Florenceville. The effluent from the McCain plant received primary treatment, i.e., a holding tank to remove larger particles by natural settling before discharge. The effluent was high in starch and contained many microorganisms which degraded the nutrients in the non-aerated tank (Lindley, 1973).

The objective of this study was to determine the efficacy of using numerical taxonomy to detect population shifts in the aerobic heterotrophic river bacteria. The sampling sites were selected to show the influence of heavy organic loading on the bacterial flora.

The study of the impact of the McCain plant effluent on the predominant heterotrophic river bacteria has been divided into two sections. This, the first report includes the test results of all of the February and September isolates and relates the test responses which are physiological and nutritional to the river location and season. The second report details the numerical taxonomy.

In order to study the effects of major environmental change on the bacterial population, we sampled in September, February, June and August. The temperature of the water ranged from 0°C under ice cover to 26°C in the summer. In addition to temperature and pH, there were many chemical and physical changes to be included in the interpretation of the population shifts; however, the selection of temperature as a major physical parameter decreased the complexity of the analysis in the initial investigation. It was shown by Chalifour & Holder-Franklin in the preceding report that temperature is the predominant controlling factor.

The preliminary study by Chalifour (1975) characterizing these bacteria provided certain information on the types of bacteria which were predominant in the river. The results demonstrated that the predominant heterotrophic bacteria were responding to changes in the environment. To expand our information, an approach was required which provided an extensive data base including many fundamental metabolic characteristics of the isolates. The methodology of numerical taxonomy was selected for this study.

The techniques of numerical taxonomy have been used very successfully to define taxa for several genera of bacteria which have been difficult to classify by the determinative method (Colwell, 1970). When this study was initiated, the use of the method had been confined to groups pre-screened by the determinative method or isolated from the environment on selective media.

In keeping with the master plan, the second phase of the study of the bacteria using numerical taxonomy to study the isolates in the river was initiated. The four seasonal samples were obtained from three stations: at the point source of the effluent of a food-processing plant, 1.6 km above and 1.6 km below the point source. The stations were selected to determine the impact on the bacterial population of the heavy nutrient outflow from a primary treatment tank and the human sewage which entered the river untreated.

The isolates obtained were characterized by numerical taxonomy. It was necessary to modify the existing methodology so that a sufficient number of discriminating tests could be utilized to include the wide range of organisms which were present in the four seasons. The group selected for study was the predominant heterotrophic bacteria, which in turn were defined as those isolates which cluster in groups of two or more when analyzed by numerical methods. Only the clustered strains were selected for this study. The results of the tests were employed to detect population shifts and to characterize the potential metabolic activities of the bacteria in the river by an analysis of the test responses. In other words, the results generated by the numerical taxonomy were extended far beyond the identification of species and demonstrated the utilization of taxometric data in the study of microbial ecology.

First Stage of Numerical Taxonomy: objective testing

At the outset, it was apparent that the classical procedures of numerical taxonomy required modification in order to process the large number of diverse strains. Numerical analysis was performed first on tests in the following categories:

a) Substrate utilization
b) Nutritional requirements
c) Metabolic end products
d) Response to physical and chemical changes

Second Stage of Numerical Taxonomy: subjective testing

The clusters of strains showing 75% or greater homology were then examined for morphological similarities. In other words, the more subjective tests were performed only on strains with a high degree of homology by the clustering criteria. This procedure accelerated the processing of the isolates considerably. In addition to the time gained by this method of analysis, greater accuracy in clustering was achieved. The newly isolated bacteria were not stable morphologically, and even under carefully controlled conditions, changes in gram-staining, size, shape, flagella and colour were observed. These results and those of the third stage are included in the report which follows this one.

Third Stage of Numerical Taxonomy: DNA analysis

The DNA of certain clustered strains was analyzed by a method developed in our laboratory (Cashion et al, 1977), the triple column method using hydroxylapatite and two ion exchange columns. The G + C % analysis was established to substantiate the phenotypic analysis. This method was well-suited to the rapid processing of freshly prepared DNA in limited quantities from bacteria recently isolated from the environment.

Material and Methods

Samples of river water were obtained on September 18, 1973; February 22, 1974; June 20, 1974; and August 22, 1974. At each site, three 500-ml samples were collected from the near-surface waters at a depth of 0.6 m.

Station A — 1.6 km above the McCain plant
Station C — at the point of entry of the effluent pipe
Station D — 1.6 km down river from the effluent outlet

At Stations A and D in February, the ice was 1 m thick and the sample was obtained by drilling. All samples were obtained at approximately 10:30 a.m.

Temperature and pH measurements on all samples are shown in Table 6. The river temperature was 12°C in September, 0°C in February, 17°C in June, and 23°C in August. The pH of water at the effluent pipe was always higher than samples above and below the effluent pipe. This reflects the high pH of the waste in the primary treatment plant, which is approximately 11 for most of the year (Table 6).

Table 6. Temperature and pH of water samples

Date	Station in relation to outlet	Water Temperature (°C)	Water pH
September, 1973	above	12.5	7.7
September, 1973	at	12.5	8.3
September, 1973	below	12.0	7.7
February, 1974	above	0.0	7.0
February, 1974	at	9.0	10.5
February, 1974	below	0.5	7.1
June, 1974	above	17.0	7.0
June, 1974	at	17.2	9.8
June, 1974	below	16.5	7.3
August, 1974	above	23.0	7.4
August, 1974	at	24.1	8.3
August, 1974	below	23.0	7.2

RESULTS

1. Environmental Parameters

According to the reports received from the Water Quality Branch, Moncton, New Brunswick, the water temperatures at the Naquadat Monitoring Station rapidly decreased from 22°C to 12°C during September, 1973; the pH of the river water, however, remained almost constantly at 7 during the same time period. The water temperatures decreased slowly to 1°C from December to January, and reached almost 0°C in February, 1974, under 1 m of ice. During the winter season, the pH decreased to

6.2 - 6.7. These reports also supported data shown in Table 6 which were obtained at the time of sampling. The temperatures obtained in our study were 12 - 12.5°C and 0 - 0.5°C at Stations A and D in September and February respectively. The pH was 7.1 at Station A and at Station D in September and 7.0 at A and 7.1 at D in February (Table 7).

However, the temperature and pH of the effluent were quite different from those of the river water in the February sample. The temperature of the effluent was 9.0°C compared with 0°C in the river and the pH 10.5 compared with 7.0 in the river. In September, the temperature did not change; the pH, however, was more alkaline at 8.3. The high pH was one of the characteristics of the effluent due to the alkaline treatment of the vegetables during the food processing.

2. Bacterial Counts

As shown in Table 7, counts obtained at 20°C were higher than those obtained at 4°C in September river samples.

3. Growth Factor Requirements

	% not requiring supplements	% requiring at least one	% which did not respond to these supplements
September:			
Above	60	23	17
Effluent	38	2	10
Below	57	12	32
February:			
Above	84	5	11
Effluent	28	8	63
Below	88	3	8

Some differences were observed in the growth factor requirements of the various isolates. The numbers of river strains in September requiring supplements were very similar above and below the effluent. The percentage of river strains in February which required vitamins or growth factors was much lower than that of the isolates from the sample taken at the effluent source. In February, 63% of the original effluent isolates could not grow on the supplemented minimal medium with any of the additional growth factors. This clearly demonstrated the hazards of assuming that the population being studied was not highly selected.

There was little difference in the viable counts of the February river samples between the two incubation temperatures. Counts of the river water, in our study, were higher in the winter samples. The counts of the river water at Station A and D in September at both 4°C and 20°C were similar. However, the February river water average count from Station D was lower than those from Station A at both incubation temperatures.

The length of time for incubation is shown in Table 7. Many colonies were visible after an incubation of 7 days, but 50% of the colonies from the September river water developed after 7 days.

Recoveries of viable isolates from initial plates to new plates were the highest in the February effluent sample C_2, and the lowest in the February river sample D_2. Although 60 strains were isolated from each sample, only 56 strains survived from Sample A_2 (see Table 8).

4. Substrate Utilization

As shown in Figure 12, most September river isolates (Samples A_1 and D_1) utilized less than 20% of the substrates tested. On the other hand, the February organisms were capable of utilizing 50-60% of substrates tested (A_2 and D_2). Most of the isolates in the September effluent sample utilized 11-30% of substrates tested. None of the February effluent isolates (C_2) utilized more than 50% of the substrates.

Thus, the percentage of utilization of the substrates by the bacteria was dependent on the season. However, similar results were obtained from the two Stations A and D (Figures 12) and a very different population seen in C, which suggested that the effluent was rapidly diluted by the river.

When the intragroup similarity coefficients of the clustered strains from all samples were compared with the total percentage of substrates utilized it was clearly seen (Figure 13) that S_J values were highest in those clusters where a large number of substrates were utilized.

Table 7. Environmental parameters and bacterial counts.

Name of Sample	Station	Location in relation to point source	Date	Water Temp. (°C)	Water pH	Viable Counts (CFU/ml) 4°C	Viable Counts (CFU/ml) 20°C
A_1	A	Above	Sept.	12.5	7.2	4.1×10^3	2.3×10^5
C_1	C	At	Sept.	12.5	8.3	1.2×10^5	1.5×10^6
D_1	D	Below	Sept.	12.0	7.7	5.6×10^3	2.2×10^5
A_2	A	Above	Feb.	0.0	7.0	1.1×10^6	1.0×10^6
C_2	C	At	Feb.	9.0	10.5	5.0×10^6	1.4×10^7
D_2	D	Below	Feb.	0.5	7.1	2.5×10^5	4.5×10^5

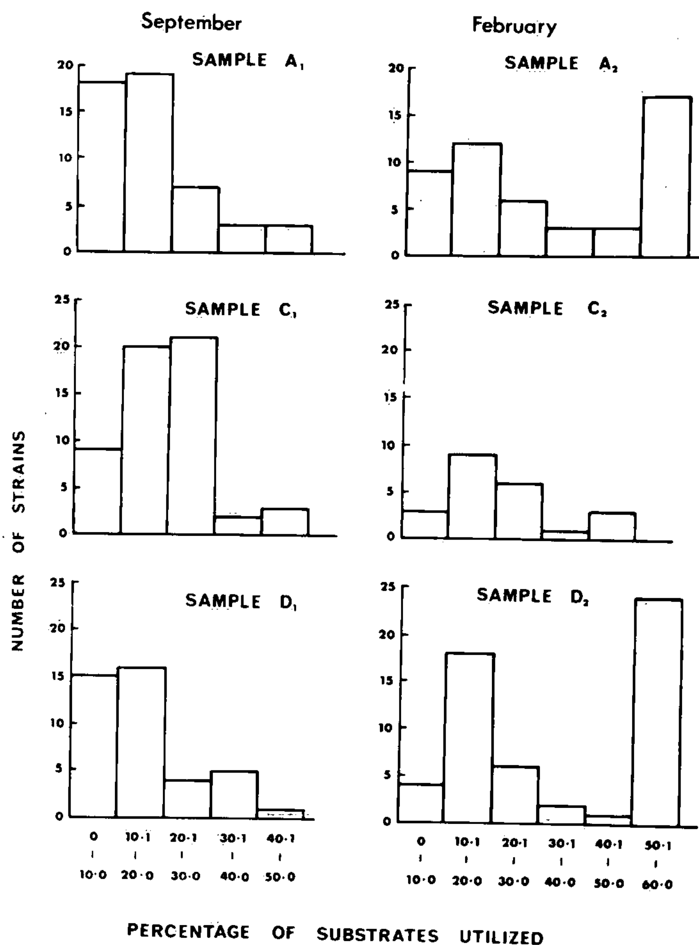


Figure 12. Percentage of substrates utilized by all isolates.

5. Utilization of Individual Substrates

The utilization of individual substrates by the river and effluent isolates are shown in Figures 14 and 15. A clear seasonal difference was seen, especially in the case of the river isolates. February river isolates utilized a larger number of substrates and were more active

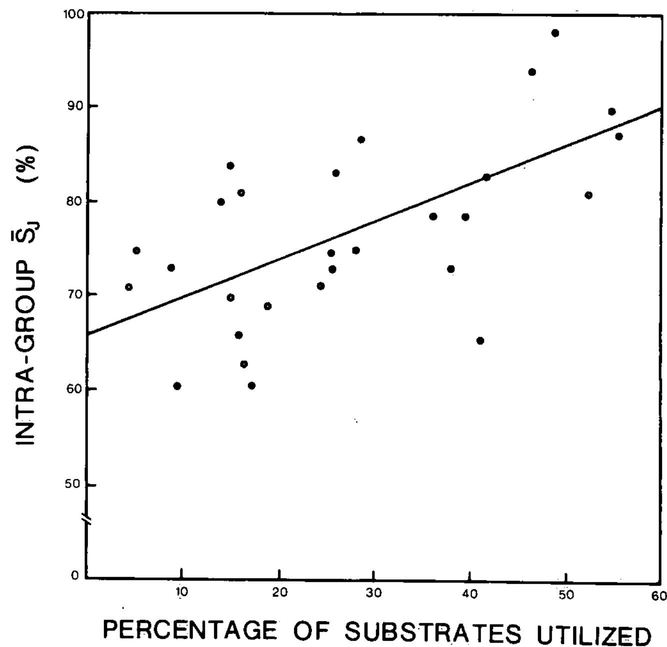


Figure 13. The intra-group similarity coefficients (%) are compared with the total percentage of substrates utilized by all clusters in February and September.

Table 8. Bacterial counts at different incubation times, and recoveries of bacteria (viable counts as CFU/ml).

Name of Sample	INCUBATION TIME (DAYS) 3 days	7 days	% increase in numbers	14 days	% increase in numbers	Recovery %	No. of Isolates
September:-							
A_1	1.2×10^5	1.4×10^5	(16.7)*	2.3×10^5	(91.7)*	87.1	60
C_1	1.3×10^6	1.4×10^6	(7.7)	1.5×10^6	(15.4)	85.0	60
D_1	1.2×10^5	1.3×10^5	(8.3)	2.2×10^5	(83.3)	72.9	60
February:-							
A_2	5.3×10^5	9.5×10^5	(79.2)	1.0×10^6	(88.7)*	74.0	56
C_2	6.6×10^6	1.3×10^7	(97.0)	1.4×10^7	(112.1)	93.8	60
D_2	1.8×10^5	4.2×10^5	(133.3)	4.5×10^5	(150.0)	68.1	60

* Shows % increase of numbers of visible colonies with increased incubation.

metabolically than September river isolates. Significant differences were seen in the case of carbohydrates, fatty acids, various organic acids and amino acids. September river isolates used few substrates with the following exceptions: D-fructose, maltose, succinate, fumarate, poly-β-hydroxybutyrate, pyruvate, sorbitol, L-glutamate and aspargine. These substrates were utilized by more than 50% of the organisms isolated at both A and D Stations; D-arabitol and l-aspartate were utilized by 50% of organisms isolated from Station A only. Most of the other substrates were utilized by less than 30% of September river isolates; even glucose and acetate were utilized by less than 30% of these organisms.

Substrates utilized by more than 50% of these organisms were D-glucose, maltose, laurate, succinate, fumarate, D,L-malate, pyruvate, L-aspartate, L-glutamate, asparagine and spermidine phosphate.

6. Miscellaneous Tests

Figure 15 compares the results of the miscellaneous tests performed on 60 isolates from each sample.

Growth at Various Temperatures

More than 95% of September river isolates failed to grow at 4°C; at 30°C, however, 100% grew. More than 90% of the February isolates grew at 4°C and more than 70% of these organisms grew at 30°C. More than 50% of the September river isolates grew at 37°C, but only 10% of the February river isolates grew at this temperature.

About 55% of the February and 24% of the September effluent isolates were able to grow at 4°C. It is interesting to note that more strains from the three February samples grew at 30°C than at 4°C.

Salt Tolerance

The effects of NaCl on the growth of the organisms were dependent on the season and location of the samples. The 0.5% concentration of NaCl was detrimental to the September river isolates. The numbers of organisms which could not grow in the presence of NaCl increased with increasing concentration.

Extracellular Enzymes

Production of extracellular enzymes was less than expected. Casein and gelatin were hydrolyzed by 50-60% of the February river isolates. Few September river isolates produced these enzymes. On the other hand, 75% of the February effluent isolates and 15% of the September isolates produced amylase. In addition, 17% of the September effluent isolates produced lipase.

Catalase and Oxidase

The majority of river isolates were catalase positive. All of the September effluent isolates were catalase positive, but only 32% of the February effluent isolates produced catalase, a phenomenon which indicated a distinct seasonal difference in the effluent populations.

The low overall oxidase production was surprising, especially in the river bacteria. The presence of fluorescent *Pseudomonas* in the February river samples increased the percentage of oxidase producers in this population, but the numbers were in the 45-50% range.

Miscellaneous Tests

Since most of the September river isolates failed to grow in various standard bacteriological media such as the glucose peptone broth used for the methyl red, Voges-Proskauer test and the Hugh and Liefson tests, a comparison of the results of these was impossible in our study.

Interestingly, 50-85% of the February river isolates oxidatively utilized glucose, and 40-55% of these isolates produced diffusible fluorescent pigment on King B, but not produce pigment on King A medium. Levan production was consistently observed in this group.

A greater number of the September effluent isolates fermented glucose with a higher incidence of gas production than the February effluent isolates. More than 50% of the September effluent isolates reduced nitrate.

Commercial Media

There were clear seasonal differences in the ability of the river isolates to grow on various commercially prepared media. Trypticase Glucose extract agar (in Figure 15) was a medium on which most river isolates could grow. About 80-90% of the September river isolates could also grow on nutrient agar. In general, the September strains did not grow well on the commercial media, however, the February isolates grew on a wide variety of media. Trypticase Soy Agar at 0.1 strength and Brain Heart Infusion Agar at 0.1 strength supported growth of the September isolates to a much greater extent than full strength media.

Dye, Bile, KCN Tolerance

In general, the February river isolates were more resistant to various dyes and chemicals than the September river isolates. The same tendencies were also seen in the case of the effluent isolates. The effluent isolates were relatively more resistant than the river isolates to bile salts. Tests for the tolerance of the bacteria to 1% phenol, 10% ethanol, 0.04% tellurite and 0.008% KCN were highly selective for special groups and were generally negative in the February and September samples.

DISCUSSION

Although numerous studies on bacterial flora in selected niches have been reported by many workers, overall characterization of the predominant organisms of the entire heterotrophic bacterial community has been neglected. Jones (1970) attempted to determine the environmental parameters which influenced the bacterial population in river basins by measuring various physico-chemical as well as biological data. In studies in stream purification, the BOD or the disappearance of certain substrates have been measured to determine the biological activity (Wuhrmann, 1964).

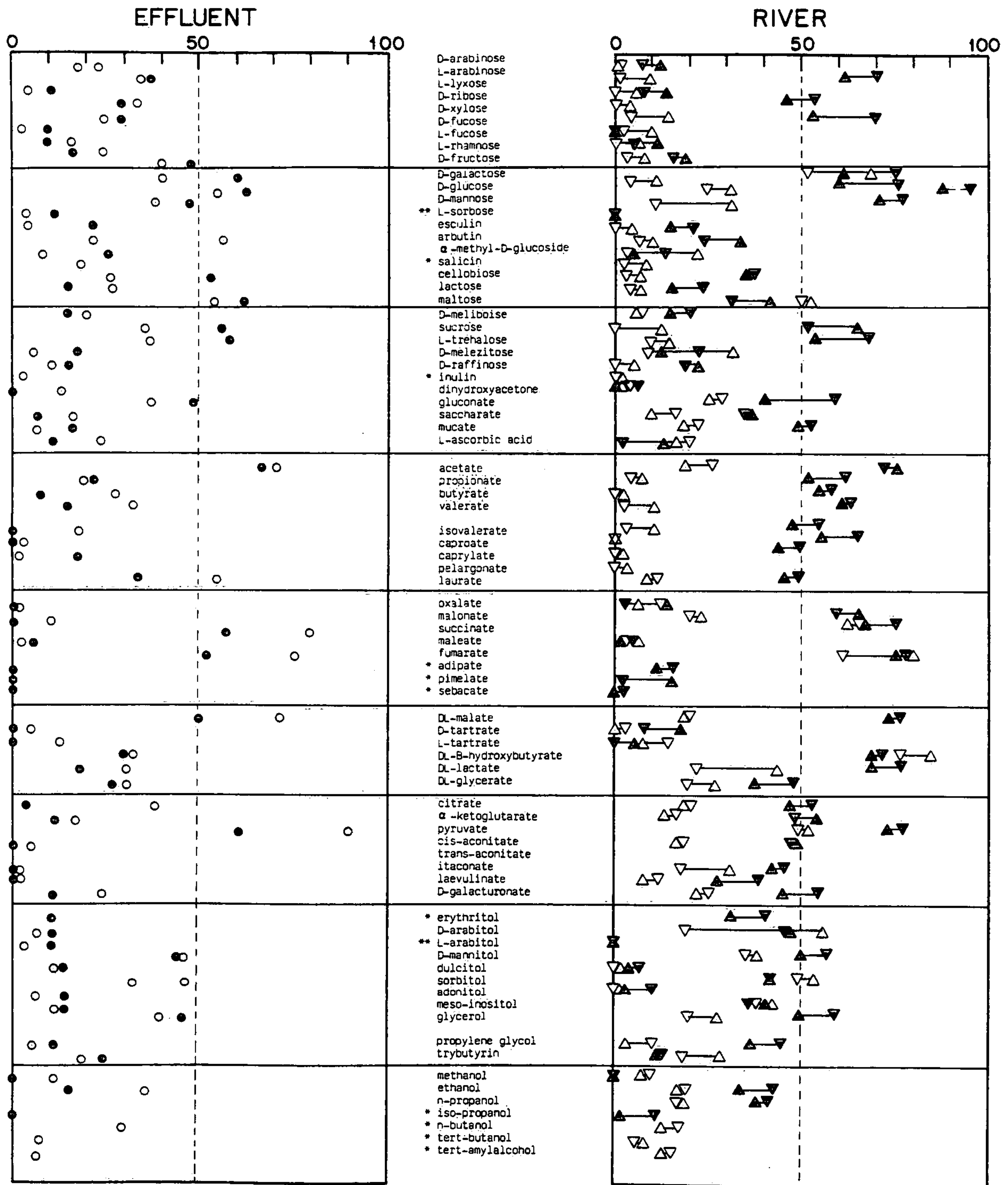


Figure 14. Percentage of isolates utilizing substrates listed.

Figure 14 continued

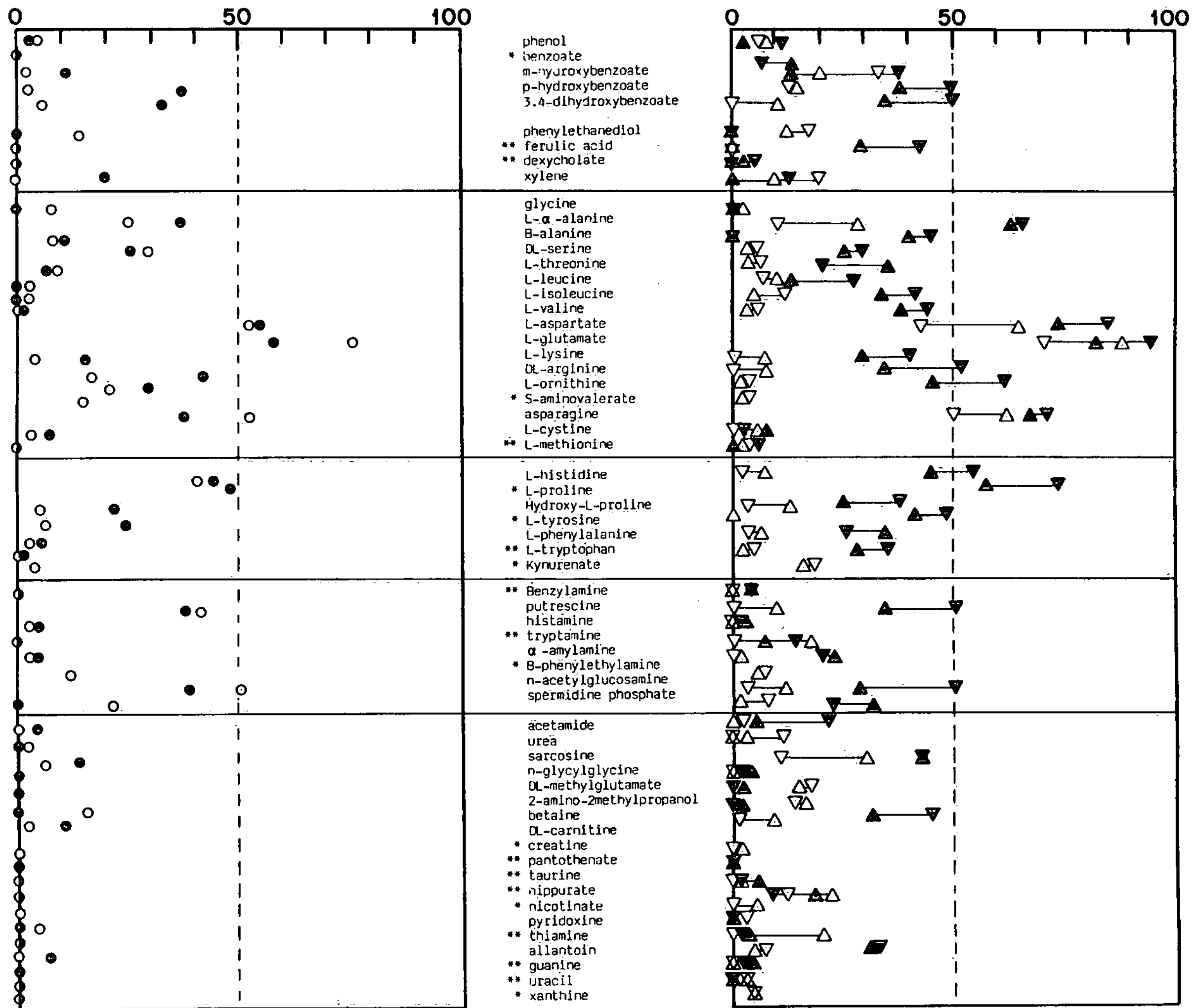


FIGURE 14 & 15 LEGEND: ▲-A_1 Sept. above, o-C_1 Sept. at effluent, △-D_1 Sept. below, ▼-A_2 Feb. above, ●-C_2 Feb. at effluent, ▽-D_2 Feb. below.

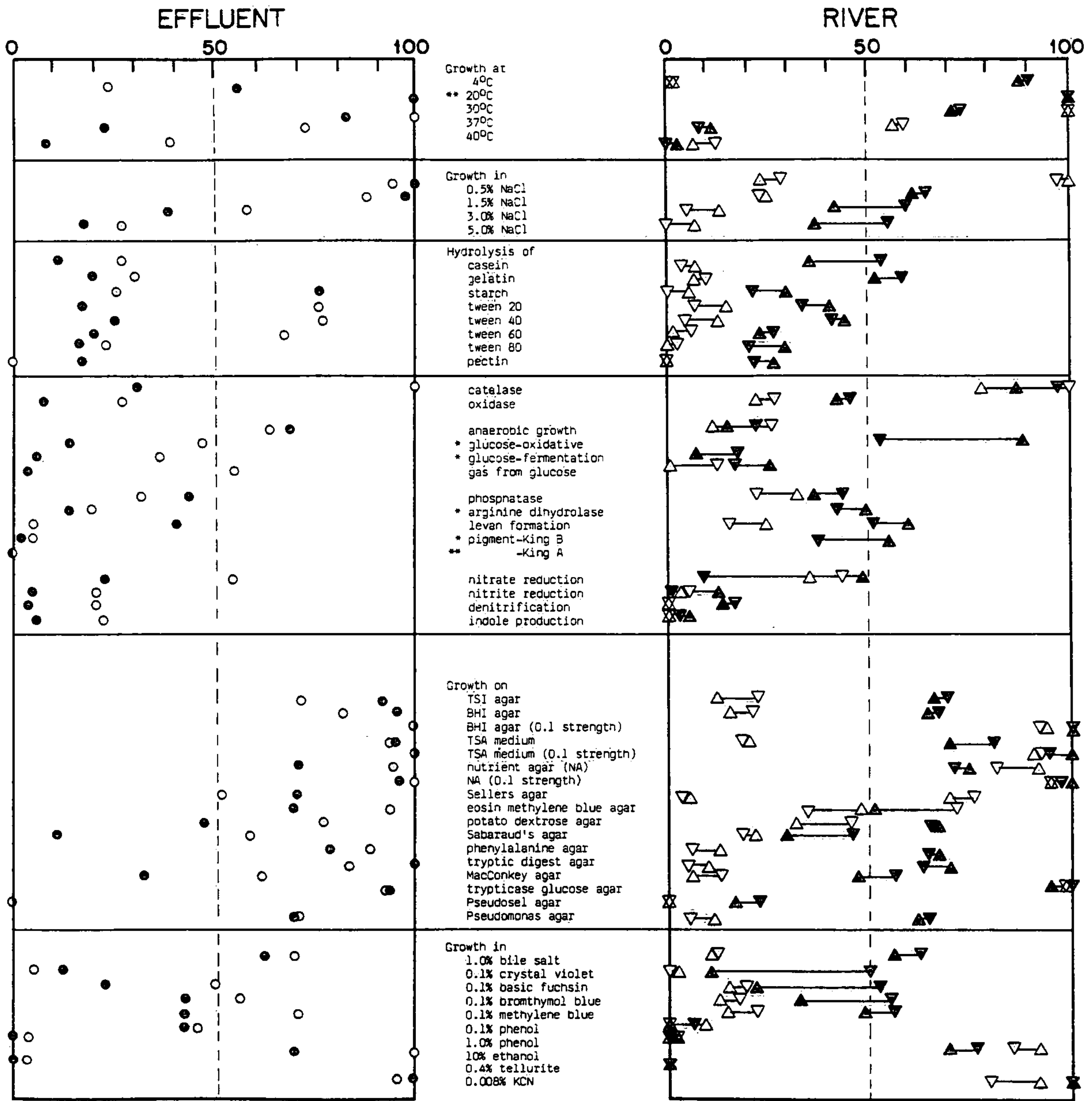


Figure 15. Percentage of the isolates positive for the tests listed.

In our laboratory, systematic microecological studies have been directed toward the investigation of the interrelationships between the bacterial community and the environment. Samples of water from the Saint John River have been obtained since 1970 (Chalifour, 1975). A constant problem encountered during these studies was the classification of the bacterial flora using the conventional methods as cited in Bergey's manual, eighth edition, (Buchanan & Gibbons, 1975). Although classification or identification of the isolates presented some problems these difficulties did not obscure the ecological interpretation of the results, i.e. an understanding of the relationships between the bacterial population and the environment.

According to the previous work in our laboratory (Chalifour, 1975), most organisms isolated from the Saint John River were gram-negative rods. Many workers have reported the same results from various freshwater environments (Collins, 1963). Bacterial counts obtained in our laboratory from the river varied between 10^4-10^7 per ml during the year; the lowest counts were in August (10^4). In this study, the viable counts of the bacteria were between 10^5 and 10^6 (Table 8). The time period for incubation was also important as were kinds of media and incubation temperatures. The incubation for enumeration of total viable counts and isolation of strains required a two-week incubation at 20°C. The recovery rate of viable strains from the river samples was 68-87%; indeed, 13-32% of the organisms were not available for the analysis. The recovery ratio must be considered in the enumeration of the total population. There was no positive correlation between low recovery ratios and incidence of slow growing organisms which were observed in the September Samples A and D, but certain correlations were observed between low recovery ratios and incidence of growth factor requiring organisms. Samples A_1, D_1, and C_2, for example, demonstrated relatively higher incidences of growth factor requiring organisms and higher recovery rates than Samples A_2, D_2 and C_1, i.e. the February river samples and the September effluent sample. One of the reasons for obtaining low recovery ratios may have been the quick death of organisms in Samples A_2, D_2 and C_1, which occurred within one week.

The majority of river organisms isolated in warmer months were known to require growth factors (Chalifour, 1975). We found that 40% of the isolates from the September river samples required growth factors and 17-32% of the September strains required unknown growth factors which we could not determine. It has been reported that isolates from rivers or sewage treatment systems such as Zoogloea ramigera required cyanocobalamin (Unz & Dondero, 1967), and many bacteria in the soil or rhizosphere require some vitamins (Lockhead et al. 1957). Bacteria such as the Enterobacter produce biotin, and the presence of biotin in rivers may be indicative of the discharge of incompletely stabilized waste (Gordon et al. 1957). However, it cannot be concluded from our data that there is a lasting influence of the effluent on the downstream bacterial community. The similarities in the populations isolated at Station D in both of the September and February studies would support this conclusion. Further studies should determine what kind(s) of growth factors are required by these organisms and whether the presence of these organisms is related to the discharge of a growth factor bearing effluent.

Most September river isolates utilized a maximum of 30% of the substrates tested; that is, the September river bacterial communities had moderate to little ability to mineralize organic substances. It is interesting to note that none of the isolates from these samples utilized more than 50% of the substrates. The incidence of organisms which utilized more than 50% of substrates was much higher in February. Although a second group in February utilizing 11-20% of substrates was also observed, these results suggested that February river isolates utilized a higher percentage of substrates, an indication of an increased ability to mineralize organic matter.

In our data, there was no evidence that the effluent communities in either September or February influenced the bacterial community of the river water with respect to changes in the pattern of substrate utilization or nutritional requirement tests. It was interesting that the utilization of substrates of the September effluent samples was not as high as that of the February samples, despite the high concentration of organic matter of the effluent. In both samples of the effluent, the organisms did not utilize more than 50% of substrates as seen in the February river samples.

The utilization of individual substrates was also characteristic of the particular sample. For example, only 20% of the September isolates utilized more than 50% of the substrates. Substrates utilized by more than 50% of the September isolates were also utilized by the February river isolates, and could be classified as universal substrates which could be utilized by most of the organisms found in the river.

The February river isolates utilized a greater variety of substrates, notably the carbohydrates, fatty acids, dicarboxylic acid and hydroxyacids. Glucose and acetate disappearance often have been employed in the study of stream purification and these substances are utilized very well by the February isolates, but at best only 30% of September isolates utilized glucose and acetate. Glucose utilization was also low in the effluent samples. Amino acids, however, such as L-glutamate, were utilized by 70-95% of all river isolates, and poly-beta-hydroxbutyrate was utilized by a greater number of February river isolates. Many September bacteria accumulated poly-beta-hydroxybutyrate in their cells as storage materials (Chalifour, 1975).

Few of the September river isolates grew at 4°C, but more than 70% of the February river isolates were able to grow at 30°C. It is reasonable to conclude that the September isolates were at a disadvantage under winter conditions. The change in population, although under the strong influence of temperature, cannot be wholly attributed to this parameter. The bacteria that predominated in February grew well at 20°C in the laboratory. The temperature of the water in summer ranged from 20°C to 25°C; therefore, it would appear that the winter flora would not be exposed to a restrictive environment. It was possible that in the summer other factors in the environment in the river were the determinative ones. Salt concentration should also be considered; at 5% NaCl, growth of 70-80% of the

September isolates was inhibited. Similarly, the September bacteria grew poorly on standard media. Problems in the identification of the organisms by classical methods were also created by the failure of most of them to grow in media such as Hugh-Leifson medium (Difco), and MR-VP medium. Many of the river bacteria were difficult to classify following the conventional taxonomy.

The influence of the discharge of starch from the food processing plant did not appear to increase the incidence of starch hydrolyzers. The February river isolates hydrolyzed more casein and gelatin than the effluent organisms and it was assumed that the mixed effluent was low in these polymers. More than 50% of the February river isolates utilized glucose oxidatively and about 40-55% of the isolates produced fluorescein.

The activities of the bacterial population have been related to the possible role of these organisms in the water habitat. Their capability for biodegradation has been inferred from their substrate utilization patterns. A general conclusion can be made that the organisms isolated from the river in February have a greater potential for a wide variety of catabolic functions than those isolated from the river in September or from the effluent. The similarity between the September isolates and the effluent isolates might imply a greater number of bacterial pollutants in the river in September.

Bacteria under the Ice

T. Kaneko, M.A. Holder-Franklin and M. Franklin

SUMMARY

All February isolates which survived the culturing procedures were analyzed by numerical taxonomy. The analysis included reference strains and effluent isolates which rarely clustered with their river counterparts. The clustered strains were identified by classical methods and the identification was supported by DNA analysis. The predominant species found at both river sites were P. fluorescens biotypes B, E and F and Flavobacterium. P. stutzeri as well as other non-speciated Pseudomonas were also observed. River clusters were identified also as Klebsiella, Aeromonas, Sphaerotilus natans and Azotobacter.

The effluent clusters, which contained only strains from the effluent, included Derxia, Aeromonas, Pseudomonas, Alcaligenes and Pseudomonas sp.

The data base of the numerical taxonomy made it possible to group strains to simplify the classification and to identify their locations. The bacteria isolated from the river two miles apart showed great similarities and the dilution of the effluent strains indicated that bacteria introduced into the river by the effluent did not become part of the free-floating population downstream. These findings supported the preliminary work of Chalifour and Holder-Franklin.

Numerical Taxonomy of February Isolates

Using the procedures outlined under methods, the February isolates described in the preceding report were clustered according to their positive responses to 250 dichotomous tests using the Jaccard coefficient S_J of Sneath (1957a) for the calculation of the similarity coefficients and the single linkage method for the clustering procedure. The matrix diagram as shown in Figure 16 is drawn to scale to show the relative sizes of the clusters . The reference strains are indicated by an inverted open triangle. Species identification of many of the isolates was not possible using the determinative method, however, the genus characteristics were clear enough to make a tentative identification. The total number of strains in the analysis was 169, including 42 reference strains. Seventy-nine strains clustered and were identified.

In further support of the need for positive results to make the most effective use of bacterial taxometrics, Figure 17 shows the relationship between intracluster S_J values using the 139 substrate utilization test base and the percentage of substrates utilized. The greater the number of substrates utilized, the higher the S_J value.

The pattern of substrate utilization varied as shown in Figure 17. The large Pseudomonas fluorescens, Biotype B cluster, and the largest Flavobacterium cluster both utilized a wide variety of substrates, however, the type of chemicals metabolized varied. The Pseudomonas group utilized fatty acids and other organic acids, whereas the Flavobacterium catabolized large numbers of carbohydrates, polyalcohols and glycols. The Klebsiella were the most limited of the selected group, utilizing only carbohydrates and aliphatic amino acids. The clusters with the lowest overall response were the Derxia and P. alcaligenes as seen in Figure 17.

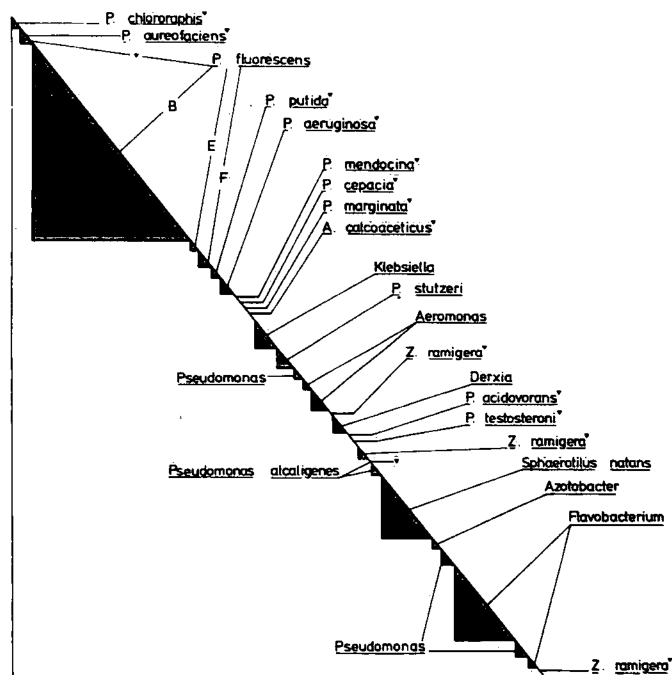


Figure 16. February matrix diagram.

▼ – after name indicates reference strain

_/ – bent line connecting name to cluster also indicates reference strains

◣ – cluster size in proportion to actual computer printout (largest cluster, 36 strains)

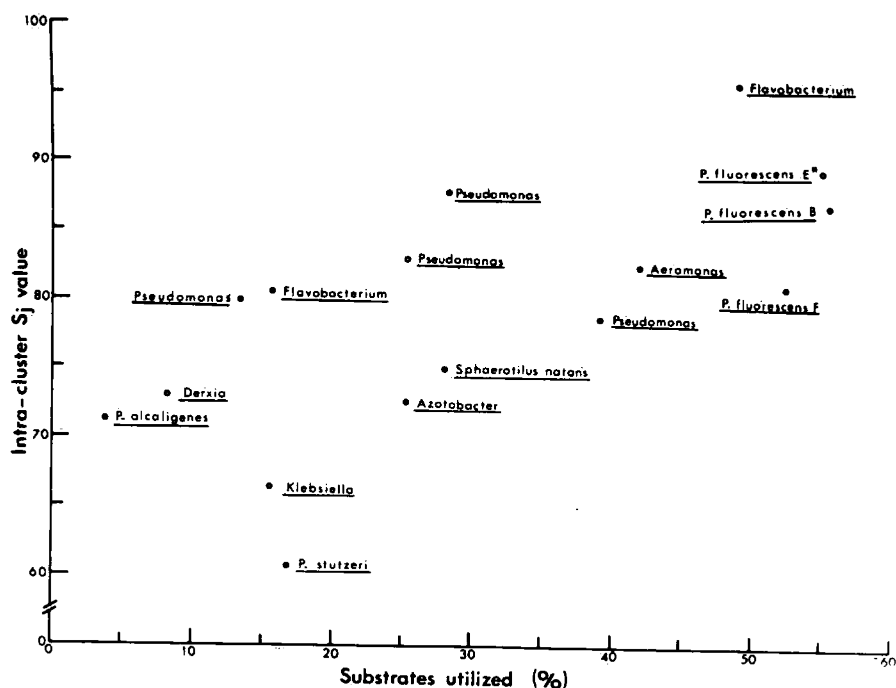


Figure 17. Relationship between similarity coefficients calculated on substrate utilization of strains within a cluster to the percentage of overall substrate utilization. Biotypes B, E, F of Pseudomonas fluorescens according to Stanier et al. (1966).

Identification of Clusters

Many determinative schemes for identification have been proposed, especially by researchers studying phytopathogenic fluorescent Pseudomonas (Lelliott et al. 1966; Schroth and Hildebrand, 1971). Unfortunately, many fluorescent Pseudomonas strains did not fit into a recognized group or species. In 1966, Stanier's group published a classic paper, an intensive taxonomic study of Pseudomonas species on the basis of the utilization of a large number of substrates as sole source of carbon and energy (Stanier et al. 1966), and the improved summary was described in the eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons. 1974). We attempted to follow this excellent guide in identifying fluorescent Pseudomonas species from river water, however, there are many Pseudomonas fluorescens strains that did not belong to any of the biotypes described by Stanier.

Pseudomonas fluorescens

Clusters Pseudomonas fluorescens Biotype B (36 strains), E (2 strains) and F (3 strains) have been selected for detailed scrutiny, as this group of fluorescent Pseudomonas predominated (top of matrix diagram, Figure 16). The intragroup similarities were greater than 75%, however, the two smaller clusters formed distinctive subclusters having very high intragroup similarities (> 80%).

Morphologically, there was little difference between organisms of these three sub-cultures. Cells were gram-negative, straight rods, 0.8-1.0 x 1.6-4.0 μm, occurred singly, motile with one polar flagellum or multitrichous with 2 to 4 flagella. Catalase and oxidase were produced. Diffusible fluorescent pigment(s) was produced on King B medium (King et al. 1954). Acid was produced from glucose aerobically. The range of temperature for growth was 4°C to 30°C and growth appeared in 0.5 to 5.0% NaCl. The enzymes caseinase, gelatinase and levan sucrase were released by Biotype B only. All organisms produced arginine dihydrolase.

Differences in substrate utilization tests between sub-clusters were seen in the following substrates (see Table 9), D-arabitol, D-xylose, L-fucose, maltose, L-ascorbic acid, malonate, itaconate, laevulinate, erythritol, sorbitol, meso-inositol, ethanol, benzoate, m-hydroxybenzoate, p-hydroxybenzoate, deoxycholate, hydroxy-L-proline, L-tryptophan, benzylamine, histamine, tryptamine, alpha amylamine, p-phenylethylamine, xanthine n-acetylglucosamine, and n-glycylglycine.

DNA analysis of eleven strains in the P. fluorescens B cluster ranged from 56.6 moles % G + C to 65.6% with a mean of 62.4 and a median of 62.2 (Table 10). Expected G + C% for P.fluorescens E is 63.6. Two of the three strains in this cluster were in the 66% range. One strain of the P. fluorescens F cluster was very close (61.9) to the expected value of 59.4%.

Table 9. Selected characteristics of species and biotypes of fluorescent, arginine dihydrolase positive *Pseudomonas*, compared with Stanier *et al*.* (1966)

	Present Study Stanier Biotypes								Stanier *et al** (1966) Bergey Biotypes [c]									
	P.fluorescens				P.chlororaphis	P.aureofaciens	P.putida	P.aeruginosa	P.fluorescens					P.chlororaphis	P.aureofaciens	P.putida	P.putida	P.aeruginosa
	B	E	F	ATCC 13525	ATCC 9446	ATCC 13985			Biotype I	Biotype II	Biotype III	Biotype IV	Miscellaneous			Biotype A	Biotype B	
P.fluorescens biotypes*				A	D	E	A		A	B	C	F	G	D	E			
Number of strains tested	36	2	3	2	2	1	2	3	24	19	15	2	17	10	7	32	9	29
Non-fluorescent pigments:**																		
Green (chlororaphin)	*d -	-	-	-	*e +	-	-	-	-	-	-	-	-	+	-	-	-	-
Orange (phenazine-1-carboxylate)	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-
Blue, non-diffusible	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Levan formation from sucrose**	*f 33	+	*g 2	+	+	+	-	-	+	+	-	+	-	+	+	-	-	-
Denitrification**	15	1	-	-	-	-	-	2	-	+	+	+	-	+	*h -	-	-	+
Carbon sources:																		
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	*i 1/-	-	-	-	-	-	-
L-Arabinose**	+	+	+	+	-	+	-	-	23	18	-	+	14	-	+	6	8	-
D-Xylose	+	-	+	+	-	-	-	1	22	15	-	1/-	13	-	-	-	6	-
L-Fucose	-	-	+	-	-	-	-	-	*j NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Maltose**	4	-	+	-	-	-	-	-	-	-	-	1/-	-	-	-	-	-	-
Trehalose**	+	1	+	+	+	+	-	-	+	+	+	+	-	+	6	-	-	-
Sucrose**	27	-	2	1	1	1	-	-	+	+	-	+	4	+	6	3	1	-
Saccharate**	24	+	1	1	1	1	+	-	+	18	-	+	14	+	+	+	8	-
L-Ascorbate	-	+	-	-	1	+	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Propionate**	+	+	+	+	+	+	+	+	22	+	13	-	14	9	+	+	+	+
Butyrate**	+	+	+	-	+	+	+	+	-	15	-	+	3	9	4	31	+	+
Malonate	35	+	-	+	+	+	-	+	+	+	+	+	+	+	+	21	+	+
Laevulinate	33	1	-	-	+	+	1	+	6	1	3	1/-	1	5	5	8	3	+
Itaconitate	34	-	+	-	+	+	+	+	22	14	6	1/-	9	+	+	6	-	+
Erythritol	+	-	-	+	-	-	-	-	20	14	+	1/-	8	4	-	1	-	-
Sorbitol**	35	-	-	+	-	-	-	-	22	+	2	+	8	-	-	1	2	-

	Present Study Stanier Biotypes								Stanier et al.* (1966) Bergey Biotypes c									
	P.fluorescens				P.chlororaphis	P.aureofaciens	P.putida	P.aeruginosa	P.fluorescens					P.chlororaphis	P.aureofaciens	P.putida	P.putida	P.aeruginosa
	B	E	F	ATCC 13525	ATCC 9446	ATCC 13985			Biotype I	Biotype II	Biotype III	Biotype IV	Miscellaneous			Biotype A	Biotype B	
Adonitol**	4	-	-	+	-	-	-	-	22	2	+	-	8	-	-	1	-	-
meso-Inositol	35	-	-	+	+	-	-	-	21	+	+	+	11	+	+	-	-	-
Propylene glycol**	35	+	+	-	-	-	+	+	-	18	12	-	8	-	-	26	-	28
Ethanol**	+	+	-	-	-	-	1	2	-	18	12	-	8	*k 1	-	31	8	+
Benzoate	-	+	+	-	+	+	+	+	8	3	6	+	5	+	6	+	+	+
m-Hydroxyoenzoate	z	-	+	-	-	-	-	-	-	-	1	1/-	-	2	-	3	-	-
p-Hydroxyoenzoate	32	+	-	NT	NT	NT	+	+	23	-	14	1/-	+	+	+	30	+	28
Deoxycholate	-	-	+	-	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Hydroxy-L-proline	32	+	-	+	+	+	+	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Tryptophan	32	1	-	NT	NT	NT	-	1	+	14	+	1/-	11	+	+	-	+	21
Histamine	-	+	-	-	+	+	+	+	13	18	-	1/-	3	9	6	+	8	+
Tryptamine	8	+	-	-	-	-	-	-	2	2	5	1/-	1	-	-	7	+	-
Benzylamine	-	+	2	NT	NT	NT	+	-	-	-	1	1/-	3	-	-	+	+	-
d-Amylamine	31	+	-	-	+	+	+	-	-	4	5	1/-	4	7	+	29	+	-
B-Phenylethylamine	5	+	-	-	-	+	+	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
n-Acetylglucosamine	31	-	-	+	+	-	1	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Glycylglycine	-	+	1	-	-	-	1	2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Hydrolysis:																		
Casein	30	-	-	-	+	+	-	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Gelatin	31	-	-	-	1	+	-	+	+	+	+	+	+	+	+	-	-	+

* Data from Tables 14 to 32 of Stanier et al. (1966)

c P. fluorescens biotypes according to Bergey's Manual (Buchanan and Gibbons, 1974)

** Differential characteristics (Buchanan and Gibbons, 1974)

*d,e,f,g; -, All strains negative; +, all strains positive; f, bold face (marked with circle in this paper) number indicates that the number represents 80% or more of the strains, plain number indicates number of positive strains.

h described as variable (Buchanan and Gibbons, 1974)

i original data meant (Stanier et al. (1966) "not positive" for two strains, therefore 1 or - were used here.

j NT - not tested

k described as "+" (Buchanan and Gibbons, 1974)

Table 10. Analysis of moles % guanine plus cytosine

Identification	Strain #	Values reported (literature) %	Obtained %
P. fluorescens B *	352	59.4 - 61.3	56.6
	323		61.3
	302		62.6
	305		65.6
	349		64.0
	318		59.9
	456		60.6
	328		62.2
	306		63.2
	354		65.22
	472		65.6
P. fluorescens E	315	63.6	66.2
	355		66.7
P. fluorescens F	458	59.4	61.9
Klebsiella	430	52 - 56	58.41
P. stutzeri	303	60.0-66.3	61.20
Pseudomonas sp. cluster a	329	59 - 68	69.7
Aeromonas sp.	451	57 - 63	61.5
	336		61.0
Aeromonas sp. # 2	368	57 - 63	59.5
Derxia sp.	388	70	67.1
	392		68.1
	382		66.6
Pseudomonas alcaligenes	394	66.3	66.7
Sphaerotilus natans	308	70.0	
Azotobacter vinelandii	356	65	59.2
	314		63.25
Pseudomonas sp. cluster b	403	59 - 68	68.0
	409		56.8
	399		63.4
Flavobacterium sp. cluster I	441		62.2
	335		55.5
Pseudomonas sp. cluster c	322	59 - 68	58.9
Flavobacterium sp. cluster II	310	69 - 70	69

* In order of appearance in the matrix.

Klebsiella

Five strains belonged to this cluster. Cells were gram-negative, short, straight rods with rounded ends, 0.8-1.0 by 0.8-2-4 µm, not motile and flagellation was not observed; catalase positive, oxidase negative, acid and gas were produced from glucose, facultative anaerobes, N_2-fixation was positive. The temperature range of growth was 4°-30°C and of NaCl concentration, 0.5-5.0%.

No growth factors were required. Average 28.3% of substrates were utilized. Fatty acids, alcohols, and non-nitrogenous aromatic and cyclic compounds, and amines were not utilized.

Organisms of this cluster were related numerically to a reference strain received as Acinetobacter calcoaceticus, however, the organisms of the cluster were facultative anaerobes and their characteristics were close to those of the Klebsiella spp. The reference strain was also a facultative anaerobe, therefore, it was possibly not A. calcoaceticus, which is described as aerobic.

Pseudomonas stutzeri and Pseudomonas sp.

Six strains belonged to this cluster, which was further divided into two subclusters, Pseudomonas stutzeri and Pseudomonas species (cluster a). Morphological and other miscellaneous results showed that there were few differences between these two subclusters. Cells of this cluster were gram-negative, straight rods, 0.8-1.0 by 1.6-4.0 µm, occurring singly, in pairs, were actively motile with polar monotrichous flagellation; catalase was positive but oxidase negative. Acid but not gas was produced from glucose, aerobically. Lipase was produced, and gelatin was hydrolyzed by Pseudomonas stutzeri only. Nitrate was reduced to nitrite, levan was produced from sucrose. The range of temperatures for their growth was 4°-30°C.

No growth factors were required by organisms of the cluster. Organisms of both subclusters utilized 40% of the substrates tested. The amines were not metabolized. Differences in substrate utilization between the two subclusters were observed in the following: L-rhamnose, D-raffinose, L-tartrate, DL-glycerate, m-hydroxybenzoate, DL-serine, L-phenylalanine and taurine. DNA analysis of one P. stutzeri strain - 61.2% as compared with the reported range of 60.0-66.3%. One strain of the Pseudomonas subcluster was 69.7%.

Aeromonas cluster #1

The two strains of this cluster were gram-negative, straight rods, 0.8-1.0 by 2.4-5.0 µm, encapsulated, motile with polar monotrichous flagellation. Acid was produced from glucose, without gas. These facultative anaerobes were catalase positive, oxidase weak or negative and Tweens 40 and 80 were hydrolyzed. These organisms grew in 0.5% NaCl, and at temperatures of 4-20°C; no growth factors were required; and 28.4% of the substrates were utilized. Alcohols, non-nitrogenous aromatic and cyclic compounds, amino acids with a ring structure, amines, and other miscellaneous nitrogenous compounds were not utilized. Both strains in this cluster were analyzed for G + C content one strain was 61% the other 61.5%. These values indicated that the organisms should be identified as Aeromonas.

Aeromonas cluster #2

Four effluent strains belonged to this second cluster of Aeromonas. Cells were gram-negative, straight, or curved rods. 0.8-1.0 by 1.6-3.0 µm, occurred singly or often in pairs, actively motile by polar monotrichous flagellation. Acid was produced from glucose, but no gas, these organisms were facultative anaerobes and catalase and oxidase positive. Casein, gelatin, starch and Tweens were hydrolyzed. Arginine dihydrolase and phosphatase were produced. Nitrate was reduced to nitrite. The ranges of temperature and NaCl concentration for their growth were 4-37°C and 0.5-3.0%, respectively.

No growth factors were required; 26% of substrates tested were utilized by all strains of this cluster. The G + C% was 59.0% for one of the strains in the cluster (Table 10).

Derxia cluster

The three strains of this cluster were isolated from the effluent and were gram-negative, small short rods, 0.8-1.0 by 1.0-3.0 µm, occurring singly, motile with polar monotrichous flagellation. Catalase and oxidase were negative and the metabolism aerobic. Acid but not gas was produced from glucose. Nitrate was reduced to nitrite. Levan was formed. The ranges of temperature and NaCl concentration were 10-30°C and 0.5-3.0% respectively. N_2-fixation was positive.

No growth factors were required; 69 substrates (49%) were utilized by the organisms of this cluster. Three strains were analyzed for G + C and were all low for this classification, 66.6-68.1%.

Pseudomonas alcaligenes

The two strains of this cluster were effluent isolates. Cells were gram-negative, slightly curved or straight rods, 0.6-0.8 by 2.0-4.0 µm, motile by polar monotrichous flagellation. Acid and gas were not produced from glucose and the organisms were obligate aerobes. Catalase variable, oxidase negative. Tweens and gelatin were hydrolyzed. The ranges of temperature and NaCl concentration for their growth were 4-20°C and 0.5-1.5%, respectively.

No growth factors were required; 20 substrates (16%), were utilized by two strains, as sole source of carbon and energy.

Organisms of this cluster were highly similar to the reference strain P. alcaligenes. The G + C% was 64, and 66.4.

Sphaerotilus natans

The third largest cluster of twelve strains were gram-negative, relatively large rods, 1.2-1.5 by 3.2-5.6 µm, often elongated (often more than 20 µm), occurring singly, in pairs and chains, were encapsulated and pleomorphic with considerable accumulation of poly-β-hydroxybutyric acid. Motility and flagella were not observed and nitrogen was fixed. Acid and gas were not produced from carbohydrates; the organisms were aerobic, catalase and oxidase positive. No growth factors were required and 17% of substrates were utilized, although polyalcohols, non-nitrogenous aromatic compound and other cyclic compounds, amines, and miscellaneous nitrogenous compounds were not utilized.

DNA analysis - 70%, G + C as expected for this group.

Azotobacter Cluster

The two strains in this cluster were gram-negative, straight, relatively large rods, 1.2-1.5 by 3.0-4.0 µm, heavily encapsulated, occurring singly, in pairs or chains. They were not motile and flagella were not observed. Accumulation of poly-β-hydroxybutyric acid was observed and colonies were of the mucoid type and were catalase positive, oxidase negative, lipase positive, and N_2-fixation positive. Growth was observed at 10-20°C and 0.5-1.5% of NaCl. Growth factors were not required. About 26% of substrates tested were utilized by these organisms as the sole source of carbon and energy. The G + C% was 59.2 and 63.25 for the two strains in the cluster (Table 10).

Pseudomonas sp. cluster b

Three strains belonged to this cluster. All were the effluent isolates. Cells were gram-negative, straight rods, 0.6-0.8 by 1.0-3.0 µm, occurred singly and often in pairs, motile with polar monotrichous flagellation. Aerobes, no acid and no gas were produced from glucose. Catalase positive, oxidase negative. Light orange pigment was produced, phosphatase positive, starch was hydrolyzed.

No growth factors were required. A relatively small number of substrates (14%) were utilized by these strains. The G + C% of the three strains was 56.8%, 68.0 and 63.4. It is possible that the organisms of this cluster are three different species of Pseudomonas. The most likely speciation for strain #403 (68.0% G+C) is P. vesicularis.

Flavobacterium

The second largest cluster, fourteen strains, was gram-negative, 0.5-0.8 by 1.6-2.4 μm, and occurred singly or in long chains; the size and shape of the cells varied considerably, one end of the cell was tapered, the other end was rounded. Motility and flagellation were not observed. A non-diffusible yellow pigment was produced. Small amounts of acid were produced from glucose aerobically, but not gas. Gelatin and starch were hydrolyzed, catalase was variable, oxidase negative. Growth was observed at 4°-20°C and 0.5% NaCl was required.

No growth factors were required and a relatively small number of substrates (16%), carbohydrates and aliphatic amino acids were utilized by more than 80% of the organisms which were identified as Flavobacterium. Two strains were analyzed for moles % G + C. One was 62.2, which placed it in the F. capsulatum species, and the other 55.5%, which is out of the Flavobacterium range.

Pseudomonas cluster c

The three obligate aerobes in this cluster were motile with polar monotrichous flagella; were oxidase negative and catalase positive. These strains utilized a limited number of substrates, did not denitrify or utilize glucose; did not require growth factors. The G + C was 58.9 moles %. The organisms in this group did not fit the description of any one species and have been placed in the Pseudomonas genus.

Flavobacterium cluster II

Two strains belonged to this cluster. Cells were gram-negative, slender, straight rods, 0.4-0.5 by 2.4-6.0 μm, occurring singly, in pairs or in chains, non-motile, and flagella were not observed. Yellow non-diffusible pigment was produced. These organisms were facultative anaerobes, catalase negative, oxidase negative, gelatin was hydrolyzed and levan was formed. Organisms grew in 0.5% NaCl, and at a temperature range of 4°C to 20°C.

No growth factors were required and a very limited number of substrates (4.0%) were utilized. They were D-glucose, D-mannose, sucrose, acetate, and L-glutamate.

Organisms of this cluster belonged to the genus Flavobacterium, and probably to the species rigense. The G + C content of 69% would support this speciation.

DISCUSSION

The predominance of fluorescent Pseudomonas during the winter season supports the findings of Chalifour (1975). According to Chalifour, 44-84% of the total bacterial population of the river in winter belonged to Pseudomonas species, and 54-94% of them were fluorescent Pseudomonas. In the study reported here, a total of 76 fluorescent strains were identified in a total population of 300 isolates and were intensively studied taxonomically. To identify these fluorescent Pseudomonas, various identification schemes were utilized (Lelliott, et al. 1966; Schroth and Hildebrand, 1971; Stanier et al. 1966). Forty-seven strains were classified as the intermediate type between P. fluorescens and P. putida, on the basis of the characteristics, gelatin hydrolysis, levan production, utilization of trehalose and mesoinositol. Twelve strains were identified as P. fluorescens, three strains as P. putida, three strains tentatively as P. marginalis, one strain was suspected to be P. cichorii, and three other strains as Pseudomonas species.

It is well known that the P. fluorescens are common in soil and water, however, information on the taxonomy of the organisms as well as quantitative and qualitative ecological data is very limited. Often they are recorded as fluorescent pseudomonads in the broad definition. On the other hand, P. aeruginosa has been relatively well studied, at least for its incidence in rivers or sewage because of the public health importance of the organisms (Headly and McCoy, 1966; Ringen and Drake, 1952).

The predominant winter bacteria, the fluorescent, Pseudomonas, formed the largest cluster (the intra-cluster S_J value was 86.7%). The three subclusters are presented as separate clusters (Figure 16). Subcluster P. putida was the lowest inter-subcluster S_J value with subclusters P. fluorescens E and F, however, the inter-cluster S_J value between the latter two subclusters was rather high (74.4%). The level was rather critical, if 75% of the S_J value was regarded as the "species level" grouping (Colwell and Liston, 1961). When the similarity values (S_J) of these three subclusters were compared with those of the arginine dihydrolase producing fluorescent Pseudomonas reference strains used in this study (Appendix A), the subcluster P. fluorescens B was the most similar (70-72% S_J) to P. fluorescens A and P. aureofaciens E. The subcluster P. fluorescens F was at the 76% level (S_J) value with P. aureofaciens and 73% with P. chlororaphis 76% with P. aureofaciens and 70% with P. putida (Biotype A), and the subcluster was not highly similar to the reference strains.

If the results on 105 substrates were compared to those of Stanier et al. using the three subclusters of arginine dihydrolase positive, fluorescent pseudomonads, 80% of the strains were in agreement if the subclusters were designated P. fluorescens biotype B (73%) and P. putida biotype B (87% S_J value).

Comparing the characteristics of the organisms isolated with those proposed by Stanier et al. (1966) for the differentiation of biotypes of P. fluorescens, P. chlororaphis (as biotype D) P. aureofaciens (as biotype E), it is clear that three subclusters obtained in this study could not be placed into biotypes described in Bergey's Manual, table 7.2 on page 221). When examining the large cluster P. fluorescens B as shown in Table 9, there are differences in the following characteristics - utilization of sucrose, saccharate, butyrate, adonitol, propylene glycol,

ethanol, laevulinate, and α-amylamine - between subcluster P. fluorescens biotype B and A. In addition, several of the carbon sources were not utilized as reported for Biotype A, P. fluorescens biotype B and P. aureofaciens, specifically utilization of sorbitol and adonitol and poly-beta-hydroxybutyrate. According to the biotypes proposed by Stanier et al. (1966) the cluster of river isolates eventually was designated biotype B, based on the lack of the characteristic non-fluorescent orange pigment.

Classification of the organisms of the small cluster P. fluorescens E was extremely difficult. The results of the substrate utilization tests showed that this cluster was closest to P. aureofaciens with an inter-cluster S_J value of 76%. On the other hand, the subcluster demonstrated a very high inter-cluster S_J value, 87% with P. putida biotype B and 84% with P. putida biotype A, on the basis of a comparison with the results of Stanier et al. (1966). The cluster P. fluorescens E did not hydrolyze gelatin, and this characteristic has been used for the differentiation of P. putida from other arginine dihydrolase positive fluorescent pseudomonads (Buchanan and Gibbons, 1974; Schroth and Hildebrand, 1971). The large cluster P. fluorescens B, and the smaller P. fluorescens E did not produce the orange or green phenazin pigments. The DNA analysis of the river P. fluorescens E was 66% and indicated that the isolates were closer to P. aeruginosa. The reported DNA analysis of P. putida of 60-63% also would not encourage us to name these organisms P. putida, although very high S_J values were obtained in the Stanier et al. (1966) comparison. Several strains did not hydrolyze gelatin, including five strains of subcluster P. fluorescens B and all strains of subclusters P. fluorescens E and F. Therefore, it is doubtful that all strains which did not hydrolyze gelatin but which were arginine dihydrolase positive within the fluorescent pseudomonads should be classified as P. putida as shown in the 8th edition of Bergey's Manual.

Subcluster P. fluorescens F had relatively low inter-cluster S_J values when compared with all of the reference strains, and the pattern of substrate utilization was quite different from all other arginine dihydrolase positive fluorescent pseudomonads. Subcluster P. fluorescens F utilized D-arabinose, maltose, m-hydroxy-benzoate and deoxycholate, which were not utilized by most of the other February isolates. Therefore, this taxonomic position was substantiated.

Numerical analyses of these fluorescent pseudomonads on the basis of the substrate utilization tests alone was moderately successful in the separation of those organisms whose phenotypic characteristics are shared by closely related organisms, as pointed out by Sand et al. (1970) in a study on the numerical analyses of phytopathogenic Pseudomonas. In our study, the addition of another 61 biochemical and physiological characteristics to the substrate utilization tests did not improve the separation, indeed inter-cluster S_J values increased. The G + C moles % of the DNA of P. fluorescens biotypes A, B, C and G are similar to each other. Biotype F is the lowest in G + C content of all the fluorescent pseudomonads. Biotypes D (P. chlororaphis) and E (P. aureofaciens) yield a distinctly different G + C content from the other P. fluorescens group (Mandel, 1966). A study on DNA homology of Pseudomonas strains shows that the group of strains representing P. fluorescens A, B and E were heterogenous in phenotypic properties and that their DNA homology study showed high and overlapping inter-strain DNA homology values (Palleroni et al. 1972) P. fluorescens biotypes D and E were very closely related to each other comparing the numerical taxonomy with the DNA homology (Palleroni et.al. 1972). From our studies it appears that each biotype of P. fluorescens has heterogeneous phenotypic characteristics, and that many intermediate biotypes exist between P. fluorescens biotypes, P. chlororaphis and P. aureofaciens.

Although there was little difference in the bacterial species between the two river stations A and D, our results showed that the bacterial flora at station A was more diverse than that at station D. This fact was easily recognized when the percentage of the predominant organisms belonging to clusters P. fluorescens B, E and F, Flavobacterium and Sphaerotilus natans were obtained, that is, organisms belonging to those three clusters were 48% and 60% of the total isolates at stations A and D respectively. The organisms of subcluster P. fluorescens were the most predominant species at two stations, occupying, 25% and 40% of the total isolates at stations A and D respectively. Organisms belonging to the Flavobacterium cluster were 9% and 15%, and the organisms of the S. natans cluster were 9% and 12%, of the total at stations A and D respectively. Thus the composition of bacterial species at the two stations, did not show any significant difference.

Clusters which were composed of isolates from the two river stations, i.e. above and below the McCain plant, included the three P. fluorescens, B, E, and F, Klebsiella, P. stutzeri, Pseudomonas clusters a and c, the small Aeromonas cluster, Sphaerotilis natans, Azotobacter and both Flavobacterium clusters.

The effluent organisms belonged in the Aeromonas cluster #2, the Derxia, P. alcaligenes and Pseudomonas b groups.

The fact that the effluent isolates were not isolated at station D (at a point one mile below the effluent) suggested little influence of the pollution at least in relation to the bacterial flora at this station.

Numerical taxonomic techniques clustered organisms belonging to the Flavobacterium and S. natans clusters. The nutritional capabilities of these organisms were relatively limited, and intra-cluster S_J values were low, therefore, it is doubtful if these isolates would have been classified if the numerical analyses were not employed. Of the 127 isolates in the numerical study, 75 or 59% clustered and were identified.

In the study of the incidence of fluorescent bacteria in a high mountain watershed, Skinner et al. (1974) observed the relatively constant population of these organisms throughout the year (100 cells per ml) and observed in some cases that those numbers were influenced by recreational use of the land and stream flow.

There are many factors considered which influence the population of freshwater bacteria (Jones, 1971). Chalifour (1975) speculated that low dissolved

oxygen in summer river water might cause the disappearance of aerobic bacteria such as Pseudomonas fluorescens.

Pseudomonas fluorescens grow at temperatures between 4°C and 30°C, therefore, summer water temperatures of the Saint John River (about 20-25°C) should not be detrimental to the organisms. Another possibility is the amount of particulate organic matter as well as dissolved organic matter in the case of winter season. The high incidence of P. fluorescens observed in October (Chalifour, 1975) may be correlated with particulate organic matter of plant origin. Fluorescent pseudomonads are associated with soils near the plant roots, in the rhizosphere (Holdings, 1960; Rouatt and Katznelson, 1961; Sands and Rovira, 1971). Rovira and Sands (1971) observed that the high numbers of fluorescent pseudomonads were found on partly decomposed wheat straw in contact with wet soil. From these observations, it is reasonable to speculate that the fluorescent pseudomonads can proliferate more successfully in the fall and winter.

The increase in numbers of P. fluorescens may be assisted by the runoff from the land. In the winter land runoff is minimal, but we did observe considerable turbidity in the water under the ice at the time of sampling.

Seasonal and Diurnal Changes in the Predominant Heterotrophic Bacterial Species of the Meduxnekeag River

C.J. Cormier and M.A. Holder-Franklin

SUMMARY

Seasonal and diurnal changes in the heterotrophic bacterial community isolated from the Meduxnekeag River at the Environment Canada monitor, Belleville, N.B., were observed in March, July, September and October. A detail of the numerical taxonomy is presented to show the efficacy of using this method to detect population shifts. The variety of species observed in these samples indicated a complexity of the bacterial flora not observed previously. Although temperature is a highly selective environmental parameter, it was shown in this study that a very diverse population was present in September and October in samples taken two weeks apart and at the same temperature. Aeromonas, Flavobacterium and non-fluorescent Pseudomonas were predominant in September, whereas in October owing to the diurnal variation certain species predominated according to sampling time. Beginning at noon, Pseudomonas carophylli, a non-fluorescent organism, and Xanthomonas campestris were preeminent. At early evening, P. fluorescens obtained a clear majority and only two strains of P. carophylli remained. Another shift placed the Flavobacterium in greater numbers by midnight. The March samples demonstrated very low numbers clustering with great heterogeneity in the samples. The shift to fluorescent Pseudomonas observed in other winters at other sampling sites was not seen. A variety of organisms were isolated in July with a slight shift to the more fermentative groups. Diurnal variation was not as apparent in March and July.

INTRODUCTION

Population shifts in the heterotrophic bacteria of the Meduxnekeag River were examined using numerical taxonomy. Investigations of seasonal as well as diurnal changes in the predominant genera were obtained on each water sample.

The Meduxnekeag River flows into the Saint John River at Woodstock, N.B. as shown on the map (Plate 6). The headwaters of the Meduxnekeag River system are

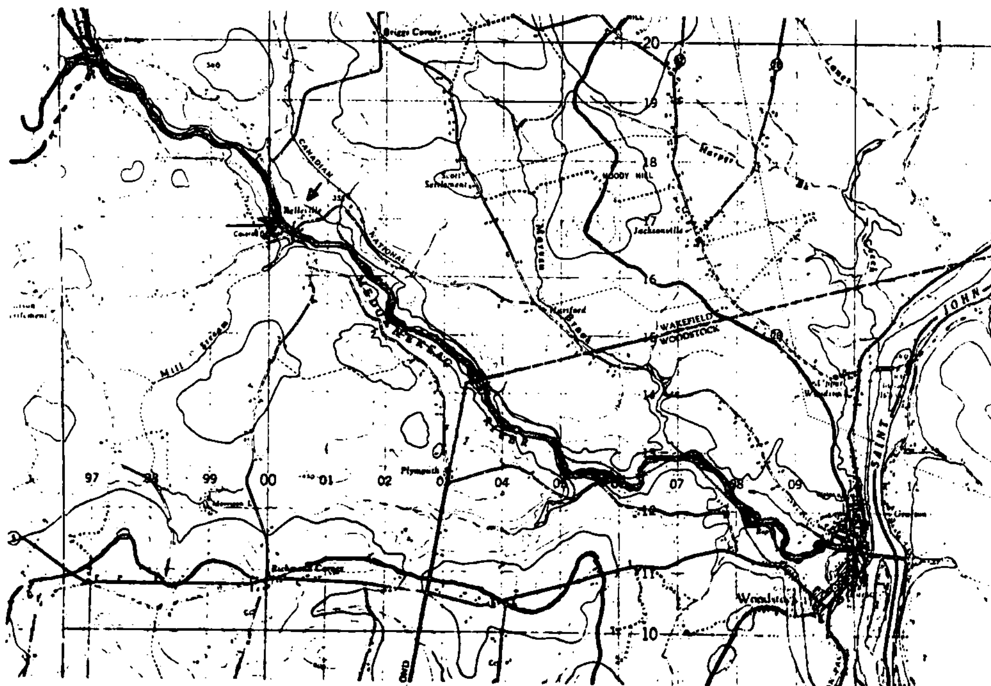


Plate 6. Map of Meduxnekeag River traced in a heavy black line which deceptively suggests that it is wider than the Saint John River.

in the State of Maine and are made up of two tributary systems; the North Meduxnekeag, and the South Meduxnekeag, which passes through Houlton, Maine. The Meduxnekeag River watershed drains approximately 1135 km^2 of land in Maine, then crosses the Maine-New Brunswick international boundary and drains 325 km^2 in New Brunswick. The principle use of the land is potato farming. Approximately two miles east of the boundary, the two branches converge and become one. The sampling site is three miles beyond the convergence in Canada. The Meduxnekeag joins the Saint John 14 miles from the border. Fishing is one of the primary recreational uses of the river because of its shallow depth, not more than 30 centimetres in many areas during summer flow periods. Other water sports are possible in and on the numerous ponds and lakes within the drainage area.

Some Sources of Pollution

A major source of pollution at the time of sampling was Morning Star Paisley, Incorporated, manufacturers of starch from potatoes. In summer, the waters were partially treated by lagooning and spray irrigation, but in winter these processes were not utilized and the effluents were discharged directly into the river system. Other sources of pollution included the discharge of sewage into the river by the town of Monticello (population 1,000) on the North Meduxnekeag and treated sewage from Houlton, Maine on the south Meduxnekeag. Private sewage disposal overflows from private dwellings along the river, as well as agricultural pollution from fertilizers, herbicides, insecticides and solids carried in land runoff storm waters. The area is sparsely populated.

During the summer of 1970, the New Brunswick Government and the Water Quality Branch, Environment Canada, installed Automatic Water Quality Monitors (AWQM) at strategic points throughout the Saint John River Basin. The objectives set for these by Environment Canada include:

1. to determine their potential as a tool in water management;
2. to measure, automatically, several water quality parameters (at hourly intervals) and to record the data in printout form for instant viewing;
3. to act as a deterrent to polluters;
4. to show water quality trends for water quality management and public information.

One such AWQM was set up at Belleville New Brunswick (see Plate 1), approximately five miles from the Maine-New Brunswick International Boundary. The parameters being monitored included water temperature, dissolved oxygen, pH, chloride ion concentration and specific conductivity. From these it was possible to see changes and fluctuations in the environment as they occurred.

A knowledge of the genera and species and the manner in which they fluctuate with environmental variables is an essential element in comprehending the role of bacteria in the aquatic habitat. Microorganisms assimilate materials directly from the abiotic portion of an ecosystem as well as from materials released by excretion or death of other organisms. This degradation of an almost unlimited variety of organic wastes by aerobic heterotrophs is of the greatest importance in the self-purification of a natural body of water. Comprehensive taxonomic data were obtained in order to relate the sensitive responses of aquatic bacteria to environmental changes as early warning indicators of deterioration or restoration in a given system.

Preliminary studies were begun in 1970, with Lindley (1975) carrying out a series of viable counts on subsurface water samples in order to determine survival patterns of the "indigenous bacterial microflora" of a selected area of the Saint John River. Then representative colonies were selected for identification and it was found that 60% of the organisms were aerobic heterotrophs. Following these preliminary studies, Chalifour (1975) isolated the predominant aerobic heterotrophic bacterial population, from samples taken in the middle of summer, autumn and winter seasons and observed a definite seasonal variation in the predominant genera. Isolates from the summer months were predominantly *Zoogloea* species and non-fermentative organisms belonging to the genera *Pseudomonas*, *Xanthomonas*, *Flavobacterium* and *Alcaligenes* in the winter. However, the determinative method of study showed the limitations of placing isolates precisely within established schemes. Many isolates, at the species level, differed in several characters from the most closely related known species. "Type Strains" from culture collections are frequently not median organisms, due to the fact that they are established with taxonomic keys using a relatively small number of biased tests.

The study to be reported here is similar to that reported in the previous paper by Kaneko *et al.* - a numerical taxonomic analysis of bacterial isolates from samples obtained diurnally and seasonally. Following cluster analysis, the clustered strains were characterized using Bergey's Manual determinative classification to determine the identity of the predominant flora.

Materials and Methods

Samples were obtained every six hours for a 18-hour period on four separate days - September 25, 1975, October 5, 1975, March 15, 1976 and July 6, 1976 - giving a total of 16 samples. The organisms were isolated on Collins agar (Appendix B). Counts were recorded on the original plates (Table 12).

Numerical Taxonomy

A total of 233 tests were performed on each isolate and the reference strains. The number of strains recovered from each sample which remained viable for testing is shown in Table 13. The computer code numbers are listed on Table 11.

RESULTS AND INTERPRETATION

Total Viable Counts

The CPS Medium of Collins and Willoughby, used in the present work for obtaining viable counts and

Table 11. Sampling dates and times, initial isolate numbers, computer isolate numbers

Date	Time	Initial Isolate Number	Computer Isolate Number
September 24, 1975	11:15 a.m.	1001 - 1100	SE 75 W 001-100
September 24, 1975	05:15 p.m.	1101 - 1200	SE 75 X 001-100
September 24, 1975	12:15 a.m.	1201 - 1300	SE 75 Y 001-100
September 25, 1975	08:15 a.m.	1301 - 1400	SE 75 Z 001-100
October 5, 1975	01:05 p.m.	1401 - 1500	OC 75 W 001-100
October 5, 1975	06:05 p.m.	1501 - 1600	OC 75 X 001-100
October 5, 1975	11:05 p.m.	1601 - 1700	OC 75 Y 001-100
October 6, 1975	07:05 a.m.	1701 - 1800	OC 75 Z 001-100
March 15, 1976	01:16 p.m.	1801 - 1900	MR 76 W 001-100
March 15, 1976	07:16 p.m.	1901 - 2000	MR 76 X 001-100
March 16, 1976	01:16 a.m.	2001 - 2100	MR 76 Y 001-100
March 16, 1976	07:16 a.m.	2101 - 2200	MR 76 Z 001-100
July 6, 1976	06:00 a.m.	2201 - 2300	JY 76 Z 001-100
July 6, 1976	12:00 p.m.	2301 - 2400	JY 76 W 001-100
July 6, 1976	06:00 p.m.	2401 - 2500	JY 76 X 001-100
July 7, 1976	12:00 a.m.	2501 - 2600	JY 76 Y 001-100

Table 12. Total viable counts of the water samples taken during the four sampling periods, in colony forming units per millilitre (cfu/ml).

Sampling Date	Sampling Time	cfu/ml on CPS media
September 24, 1975	11:15 a.m.	6.0×10^5
September 24, 1975	05:15 p.m.	1.0×10^6
September 24, 1975	12:15 a.m.	8.3×10^5
September 25, 1975	08:15 a.m.	2.0×10^5
October 5, 1975	01:05 p.m.	6.0×10^7
October 5, 1975	06:05 p.m.	1.8×10^8
October 5, 1975	11:05 p.m.	1.85×10^8
October 6, 1975	07:05 a.m.	2.0×10^6
March 15, 1976	01:16 p.m.	9.8×10^4
March 15, 1976	07:16 p.m.	1.1×10^5
March 16, 1976	01:16 a.m.	8.6×10^4
March 16, 1976	07:16 a.m.	1.5×10^5
July 6, 1976	06:00 a.m.	2.4×10^6
July 6, 1976	12:00 p.m.	8.7×10^5
July 6, 1976	06:00 p.m.	1.1×10^6
July 7, 1976	12:00 a.m.	1.1×10^6

Table 13. The total number of organisms from each of the 16 samples surviving purification.

Sampling Period	Sampling Time	Number of Organisms Recovered in Each Sample in %	Total Number of Organisms Recovered for Each Sampling Period
March	Midday	75	
	Early evening	58	265
	Midnight	60	
	Early morning	72	
July	Midday	95	
	Early evening	81	373
	Midnight	97	
	Early morning	99	
September	Midday	67	
	Early evening	77	280
	Midnight	65	
	Early morning	71	
October	Midday	97	
	Early evening	85	340
	Midnight	84	
	Early morning	71	

Table 14. The number of strains clustered for each sample, the total number clustered for each sampling period and the percentage of the original number of isolates which have clustered.

Sampling Period	Midday Samples (W)	Early evening Samples (X)	Midnight Samples (Y)	Early morning Samples (Z)	Total Number Clustered	% of the original # of isolates Clustered
March	11	9	8	13	41	16%
July	11	20	22	46	99	30%
September	49	63	38	38	188	71%
October	91	67	65	64	287	84%

purifying the randomly selected strains, was discussed by Jones (1970) and the following general observations were made. A surface spread plate on CPS medium was the most suitable for obtaining the maximum viable counts of freshwater bacteria. Total colony forming units were higher on CPS medium than on the recommended standard media for testing water quality. The colonies on CPS media were smaller and discrete, whereas on the standard media they tended to be larger and overlap, resulting in decreased counting accuracy during prolonged conditions of incubation. The smaller colonies also facilitated random selection.

Seasonal and Diurnal Variations in Bacterial Numbers

The bacterial counts on Collins and Willoughby medium from the river water samples are recorded in Table 12, together with the sampling dates and times. Variations in the total number of colony forming units per millilitre were observed seasonally, with the minimum number present during the March sample and the maximum number during the October sample. The bacterial counts from the July and September samples were very similar. A 10-fold increase in bacterial numbers was observed in the July and September sampling periods when compared with the March sample and a further 100-fold increase in October, indicating a slight seasonal shift in bacterial numbers. However, the changes either seasonally or diurnally were not sufficient to form a pattern.

CLUSTER ANALYSIS

The results of the initial UPGMA computations for each sampling period are shown in Figures 18, 19, 20

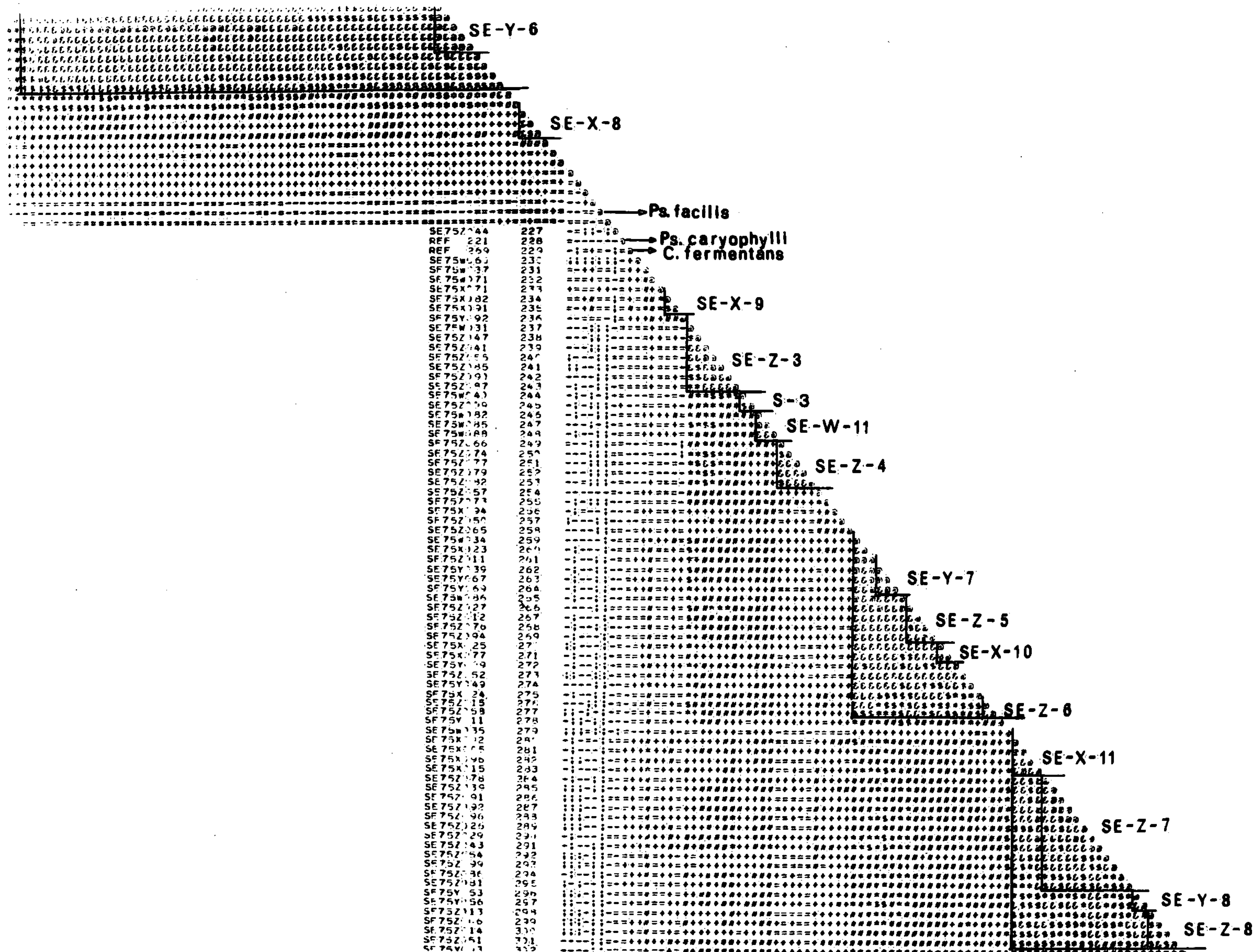


Figure 20d

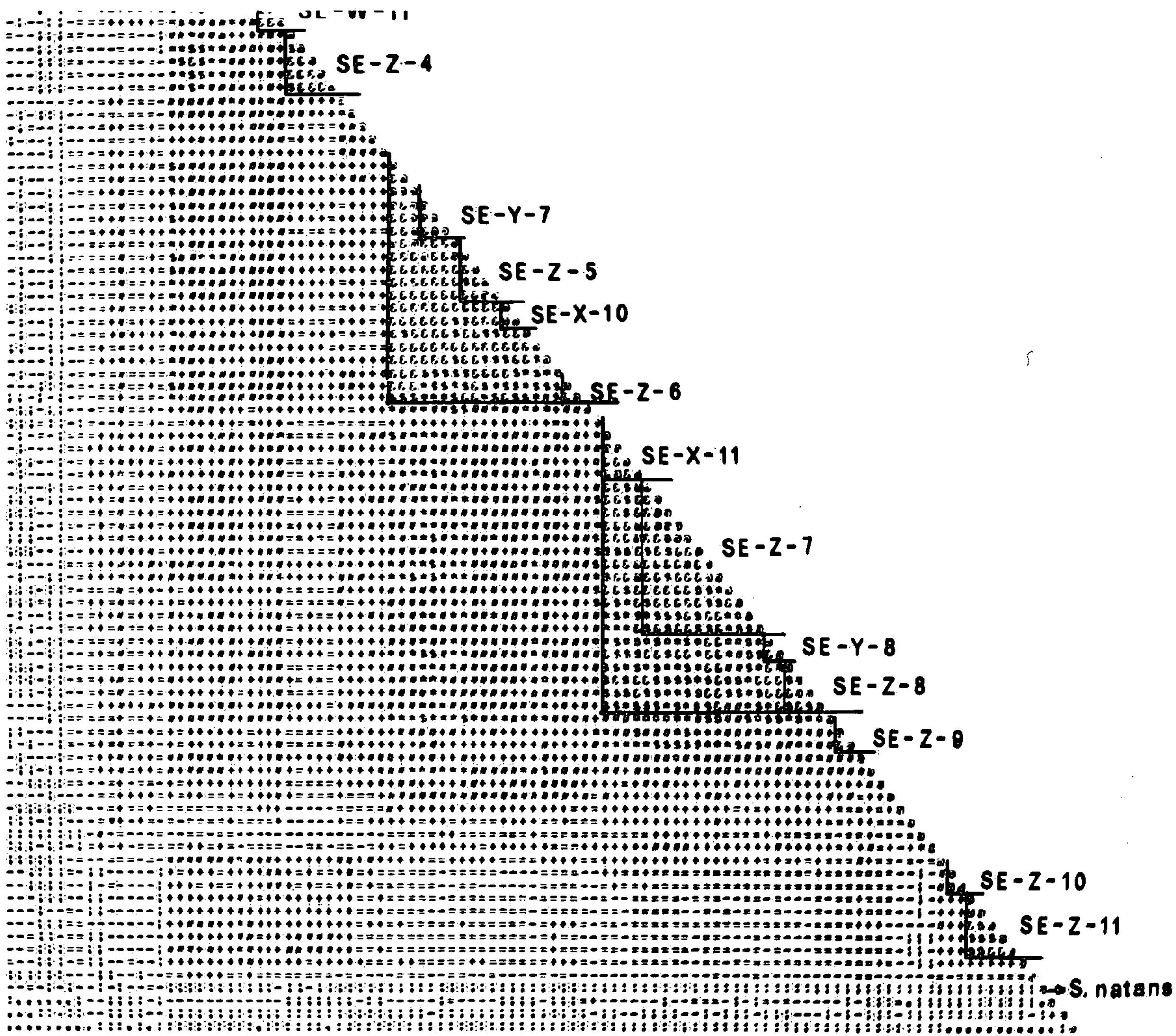


Figure 20e

Figure 21 a-f. Association coefficient similarity matrix of isolates found in the October sampling period. Shown are the reference strains and the isolates as they were clustered by UPGMA.

KEY

90 -	100 %	@
80	90 %	ε
75	80 %	$
70	75 %	*
60	70 %	#
50	60 %	+
40	50 %	=
30	40 %	-
20	30 %	;
10	20 %	:
0	10 %	.

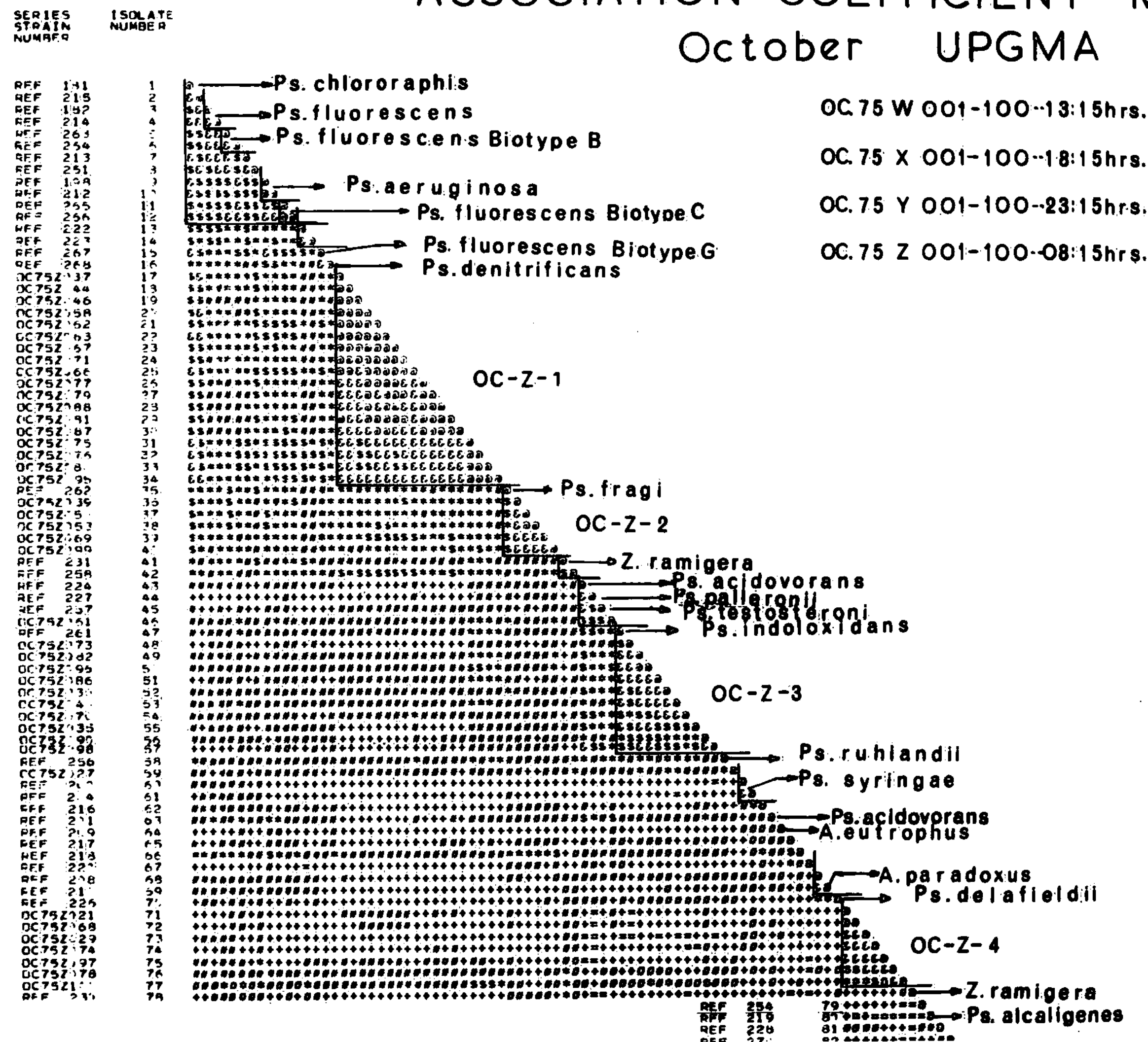


Figure 21a

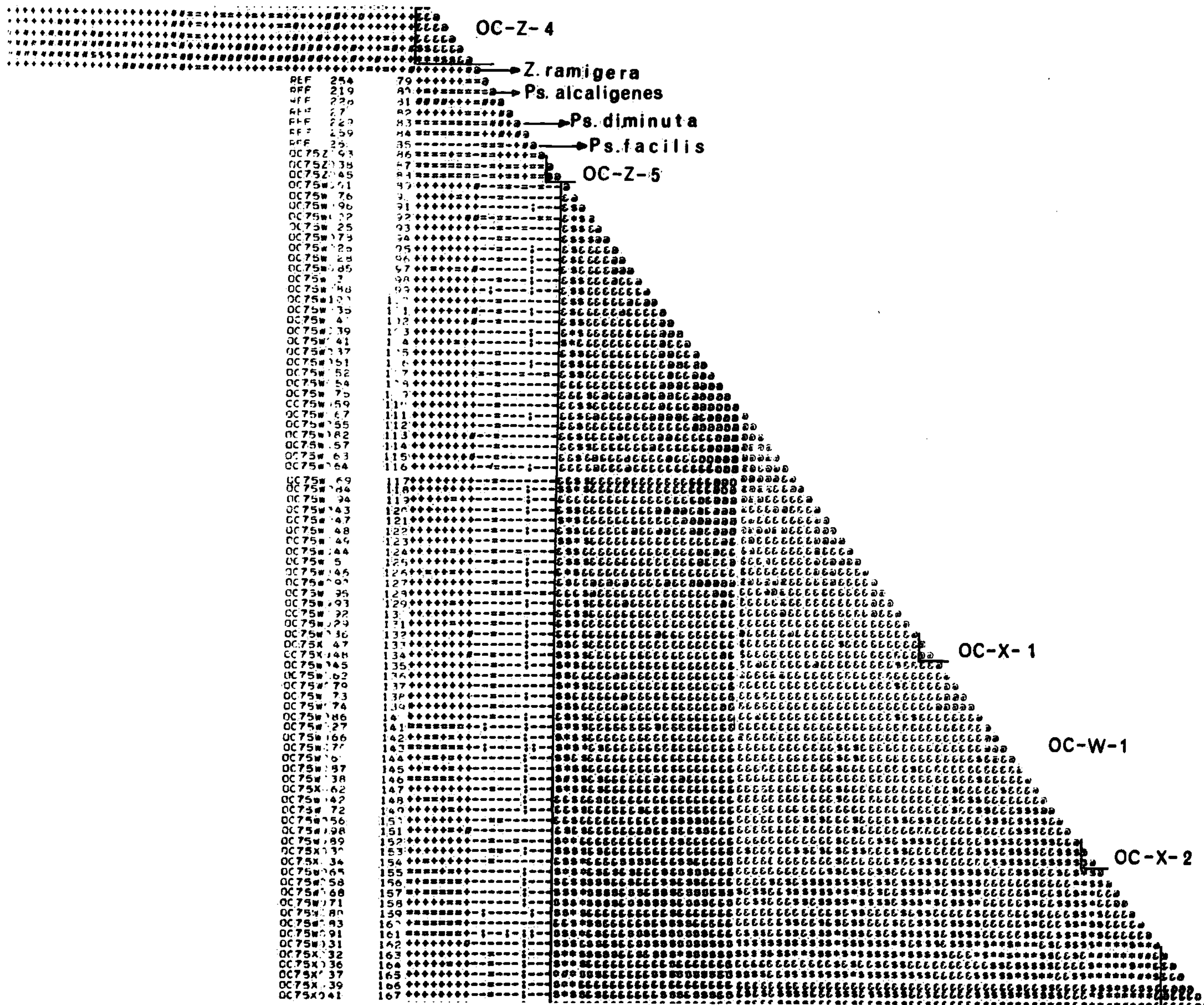


Figure 21b

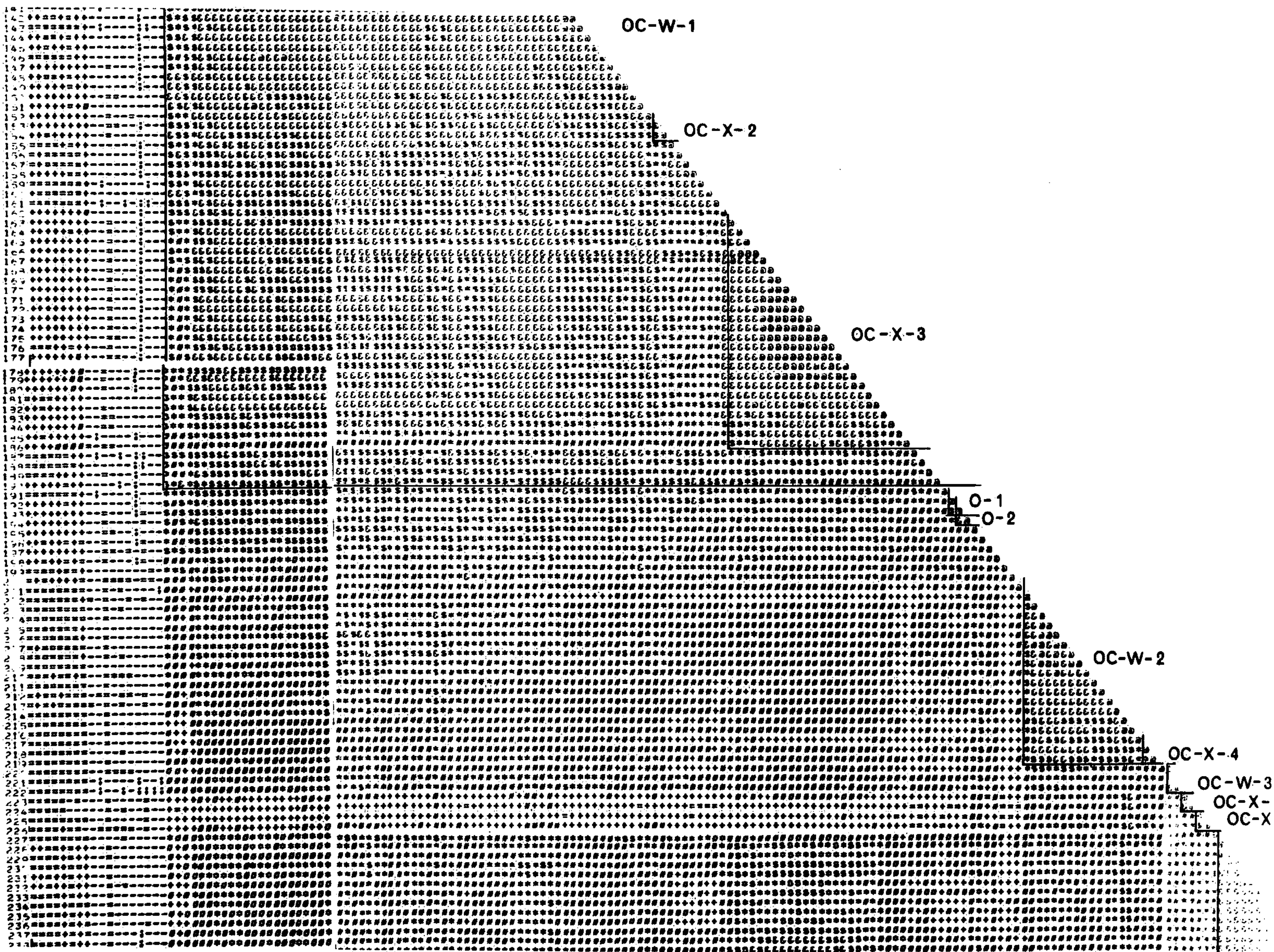


Figure 21c

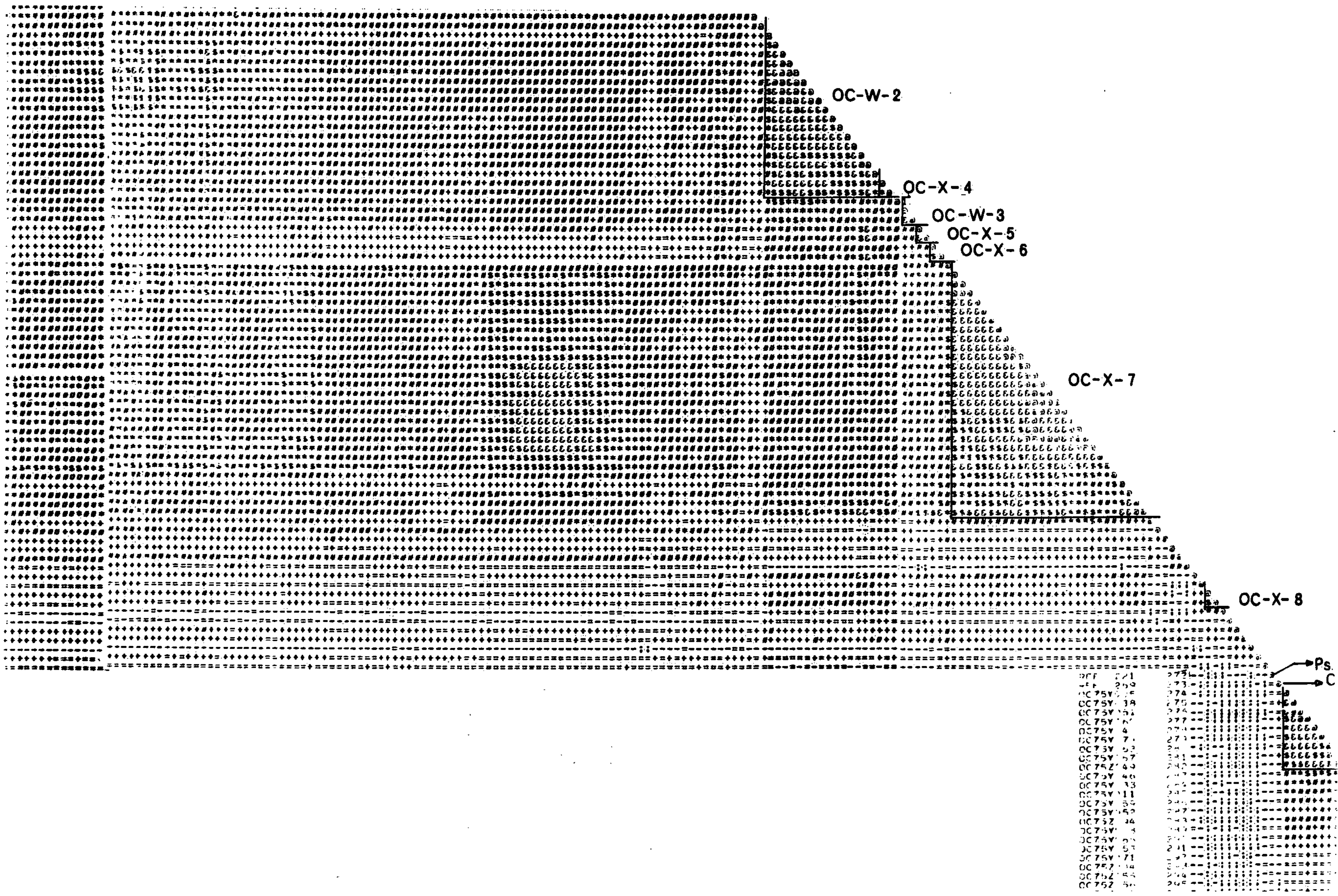


Figure 21d

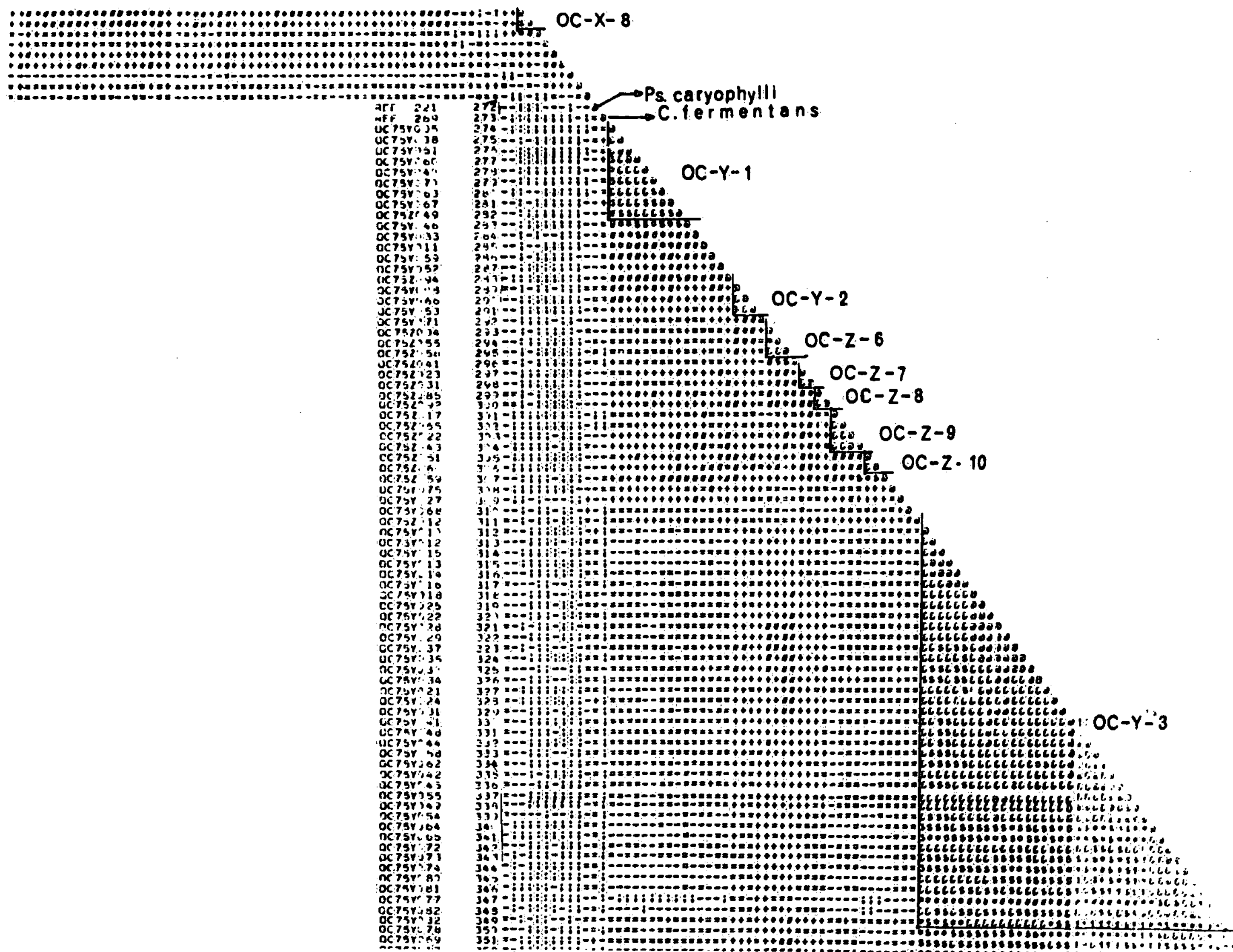


Figure 21e

OC-Y-3

OC-Z-11

OC-Y-4

OC-Y-5

OC-Z-12

OC-Y-8

OC-Y-9

OC-Y-10

S.natans

Figure 21f

Diurnal Variations of the Heterotrophic Bacteria in the Sediment and Water of a Small Woodland Stream

C.R. Bell, M.A. Holder-Franklin and M. Franklin

SUMMARY

Diurnal variation in certain biological, physical and chemical parameters was observed in water samples collected from the Dunbar River, a pristine woodland stream, periodically during a 56-hour period in August. A reduction in O_2, pH, C and P was accompanied by a decrease in chlorophyll a and bacterial counts in water and sediment incubated at 20°C. As darkness descended, the turnover time took a noticeable upswing followed by buildup in NO_3 and eventual peaking of the Vmax. Dusk also brought an increase in sediment counts at 5°C. Another peak was observed in these sediment psychrophiles at dawn. Species varied somewhat- an increase in fermentative enterics was observed during the day, with the converse true of the aerobic, non-fermentative group. The modest cycling of species in this study was also observed in the Meduxnekeag in a previous summer study.

The factor analysis demonstrated the clear differences in the sediment and water populations. Links between these populations are only speculative from the results reported here.

INTRODUCTION

Heterotrophic bacteria are the main agents of microbial degradation in many receiving waters. The indigenous bacteria in the Saint John River system have been under investigation in this laboratory since 1972 (Franklin & Coulter, 1972; Lindley, 1973; Chalifour, 1974; Olive, 1976; Cormier, 1978). In a comparative study between the Meduxnekeag River and a contrasting river, the Dunbar, little seasonal variation was observed. Diurnal and seasonal fluctuations had been observed previously in the Saint John river and its tributary, the Meduxnekeag (Holder-Franklin et al. 1978, Cormier 1978, Kaneko, 1975). Data has been scarce on diurnal fluctuations in rivers since the pioneering work of Odum (1956), although interest is beginning to revive (Kelly et al. 1974; Sieburth et al. 1977).

This paper described an investigation into the diurnal fluctuations of bacteria in the Dunbar River by the use of taxometrics, species diversity and factor analysis.

MATERIALS AND METHODS

Samples were taken from the Dunbar River every 4 hours beginning at 10:00 p.m. on 15th August 1978 through to 6:00 p.m. on 17th August giving a total of 12 samples. Water samples for bacterial enumeration were collected from the surface waters in the sterile 200-ml chamber of a Sorvall Omnihomogenizer. Water samples for assessment of heterotrophic activity were simply collected in sterile Nalgene bottles. Sediment samples were taken from the top few centimetres with the aid of a core sampler designed by Daniel & Chesters (1971).

Bacterial enumeration

Water samples were first homogenized at slow speed for approximately 5 min. Estimates of total heterotrophs, NO_3 assimilators and NH_4 assimilators were achieved using a defined glucose-nitrogen medium (Brown & Stanley, 1972) with a supplement of 0.2% w/v casamino acids, GN(cas); 200 g $N\text{-}NO_3$ ml^{-1}, $GN(NO_3)$ and 200 g $N\text{-}NH_4$ ml^{-1}, $GN(NH_4)$ respectively. Putative nitrogen fixers were estimated on the media of Norris (1959), N21. Denitrifiers and nitrate reducers were estimated on the medium of Sreenivasan & Venkataraman (1956), D30, incubated in a Gaspak jar.

Sediment estimates were performed on identical media on the basis of one gram wet weight as a slurry. Total heterotrophic counts were performed by the spread plate technique at 4°C and 20°C. An estimate of anaerobic heterotrophs was performed on a spread plate incubated in a Gaspak jar (BBC) at 20°C. All other estimates were as most probable numbers (M P N) at 20°C.

Taxometric analysis

Twenty colonies from each time sample growing on the GN(cas) plates at 20°C were picked off at random and purified. The 240 isolates were then identified as described previously (Bell, Holder-Franklin & Franklin, 1980) on a range of 82 tests in a numerical taxonomy. Isolates producing clusters at the 75% similarity level were identified by reference to the ATCC strains in the taxonomy and through Bergey's Manual of Determinative Bacteriology (8th Edition).

Mathematics analysis

The positive test response frequencies of all isolates were then examined by factor analysis (Holder-Franklin, 1980) using the Varimax method of factor rotation and NT-Z computer program. Diversity indices of the bacterial populations were calculated according to Simpson and Shannon-Weaver as mentioned in Pielou (1969). Correlations and statistical analysis were calculated via the Statistical Package for the Social Sciences (SPSS).

Heterotrophic activity

Heterotrophic activity was measured by determining the uptake and mineralization rate of uniformly labelled ^{14}C-glucose, specific activity 230 C. M^{-1} obtained from Amersham Searle, Don Mills, Ontario (Wright & Hobbie, 1966). Samples were taken back to the laboratory and incubated with shaking, at the in situ river temperature, for one hour as detailed in Albright & Wentworth (1973). Millipore and fluted filters, once dried, were immersed in vials of a PPO-POPOP Toluene mixture (Liquifluor, New England Nuclear, Boston, Mass.) and counted for 10 minutes on a Beckman LS7000 liquid scintillation counter. Quenching was corrected by the channels ration method. The data were computerized and the values Vmax, Kt + Sn, and turnover time taken from the line of best fit on a modified Lineweaver - Burk plot.

Physico-chemical parameters

Temperature and oxygen concentration were read at the site with a YSI oxygen meter (Yellow Spring Instrument Co., Ohio 45387). Chlorophyll a was determined spectrophotometrically after acetone extraction (Strickland & Parson, 1968). pH was measured in the laboratory on a Fisher Accumet pH meter. Carbon, nitrogen and phosphorous analyses were performed by the Water Quality Laboratory of Environment Canada, Moncton, N.B.

RESULTS AND DISCUSSION

Figure 22 demonstrates the reduction in O_2 saturation, pH and chlorophyll a during the night. This represented the increase in respiration during the dark with concomitant cessation of photosynthesis. Chlorophyll a peaked around midday before declining again in the late afternoon. Jackson & McFadden (1954) and Verduin (1957) noticed a similar response in chlorophyll a.

Temperature also dropped at night (Figure 23). The river exhibited a 6°C fluctuation, reaching a maximum of 18°C at midday. Estimations of heterotrophic activity showed a marked diurnal oscillation with turnover time fastest just after dusk. Allen (1971) found a greater net uptake of ^{14}C-labelled products by epiphytic micro-organisms on Najas flexilis in the dark. Apart from the first two anomalously high values, Vmax showed small diurnal variation. Percentage respiration remained at 50% throughout the study. Organic C and total P (Figure 24) showed a tendency to decrease during the night, increasing slightly during the midday. NO_3 accumulated considerably during the night and decreased in the light. This is believed to be the result of NO_3 assimilation by the algae and possibly some bacteria.

Figure 25 shows the different diurnal trends in total heterotrophic counts in the water depending on the temperature of incubation. Generally, counts at 20°C

Table 24. Mean viable counts

Media	Growth Temp.	Water	Sediment
Glucose Nitrogen + casamino acids	20°C	$4.13 \times 10^3 \pm 1.53 \times 10^3$	$2.71 \times 10^4 \pm 1.36 \times 10^4$
+ casamino acids	5°C	$7.5 \times 10^2 \pm 2.2 \times 10^2$	$5.32 \times 10^3 \pm 2.43 \times 10^3$
+ 200 µg/ml NO_3	20°C	$1.84 \times 10^3 \pm 1.76 \times 10^3$	$1.02 \times 10^4 \pm 1.01 \times 10^4$
+ 200 µg/ml NH_4	20°C	$2.32 \times 10^3 \pm 1.61 \times 10^3$	$2.36 \times 10^4 \pm 4.62 \times 10^4$
N20	20°C	$3.7 \times 10^2 \pm 3.2 \times 10^2$	$1.24 \times 10^4 \pm 3.67 \times 10^4$
N21	20°C	$3.2 \times 10^2 \pm 3.8 \times 10^2$	$1.30 \times 10^3 \pm 1.05 \times 10^3$
N22	20°C	$8.4 \times 10^2 \pm 4.6 \times 10^2$	$5.10 \times 10^3 \pm 9.53 \times 10^3$
Denitrifiers gas pack	20°C	$0.86 \times 10^0 \pm 0.78 \times 10^0$	$7.73 \times 10^0 \pm 7.14 \times 10^0$
D 30 : NO_3 reduction	20°C	$1.96 \times 10^3 \pm 1.88 \times 10^3$	$3.24 \times 10^4 \pm 4.59 \times 10^4$
Glucose Nitrate + casamino acids Anaerobic	20°C	not estimated	$3.26 \times 10^3 \pm 1.41 \times 10^3$
% Psychrophiles		17.3% ± 5.0%	16.9% ± 5.7%

Water counts given per ml; sediment counts per gram wet weight.

Media designations as explained in methods.

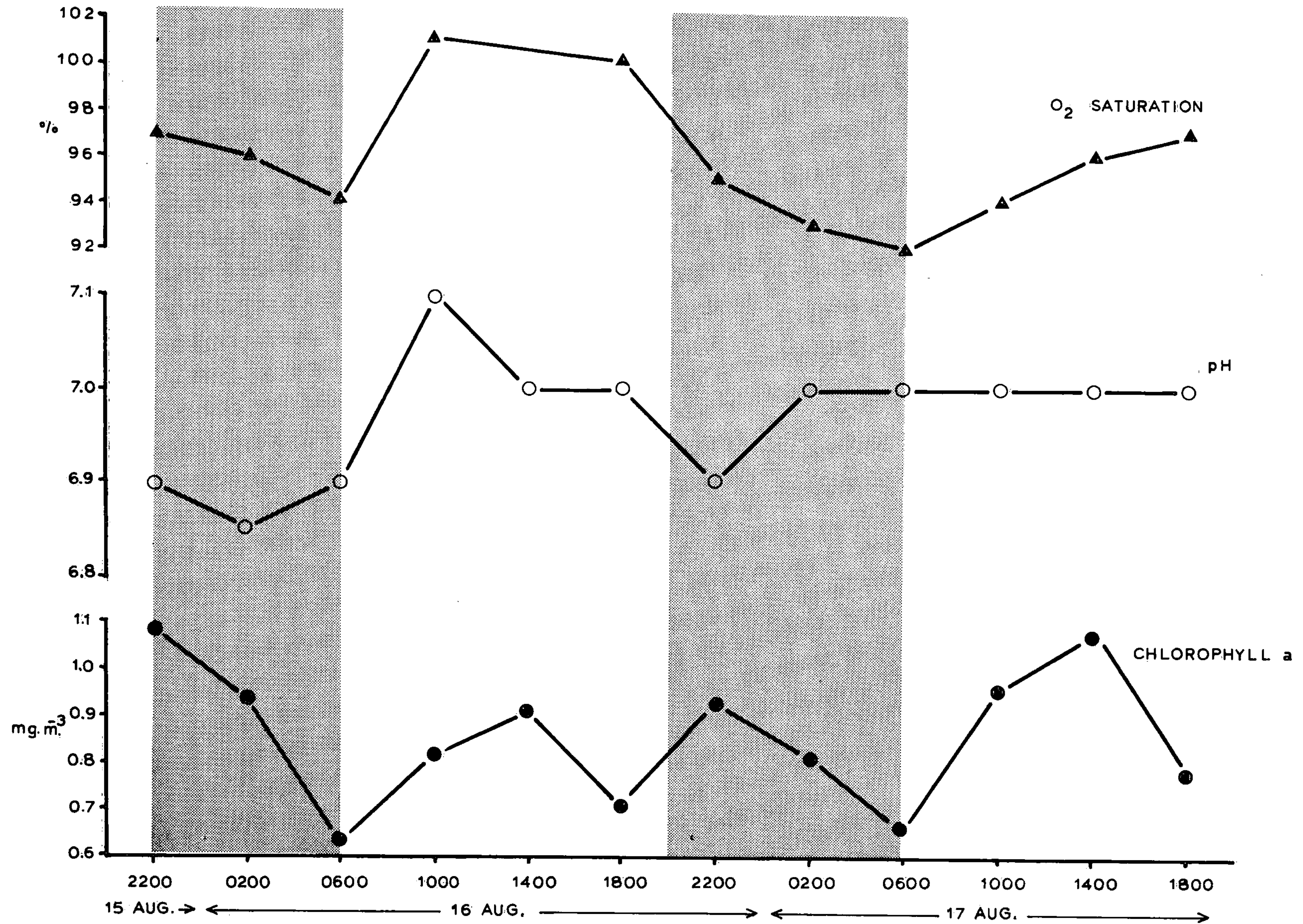


Figure 22. Fluctuations in O_2 saturation, pH and chlorophyll a. The shaded area corresponds to the hours of darkness.

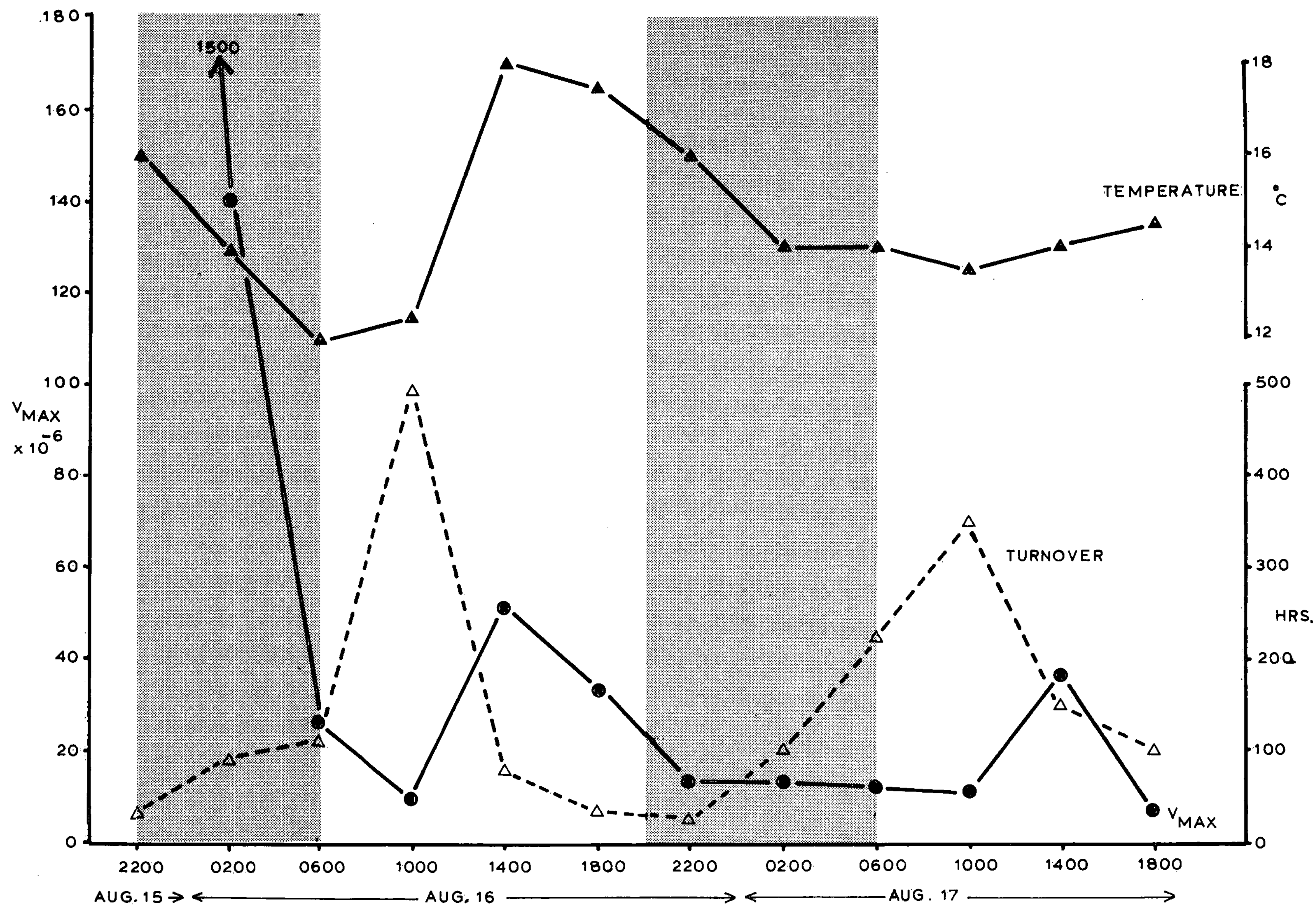


Figure 23. Fluctuations in temperature, V max and turnover time. The shaded area corresponds to the hours of darkness.

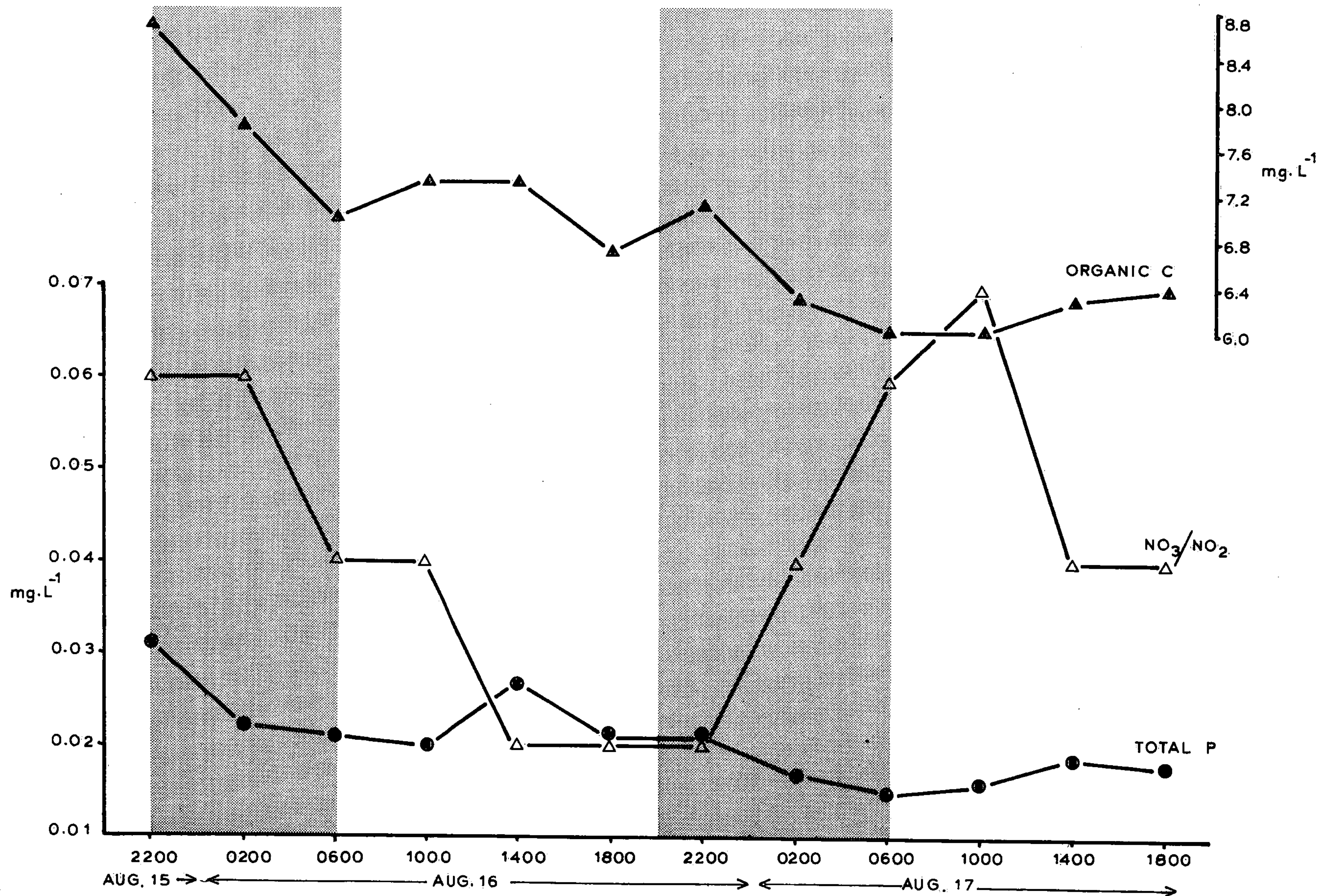


Figure 24. Fluctuations in organic C, NO_3/NO_2 and total P. The shaded area corresponds to the hours of darkness.

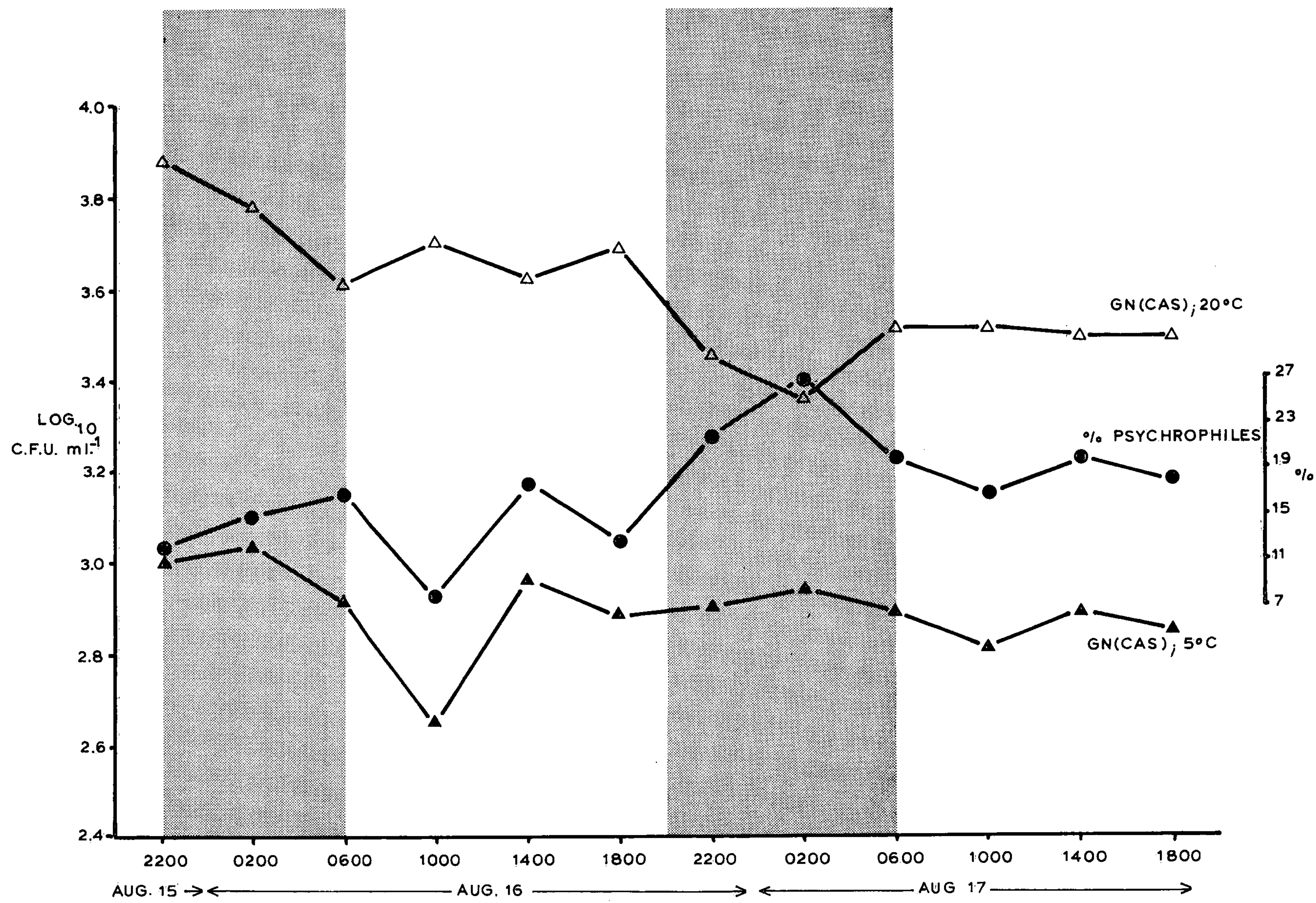


Figure 25. Fluctuations in water viable counts. The shaded area corresponds to the hours of darkness.

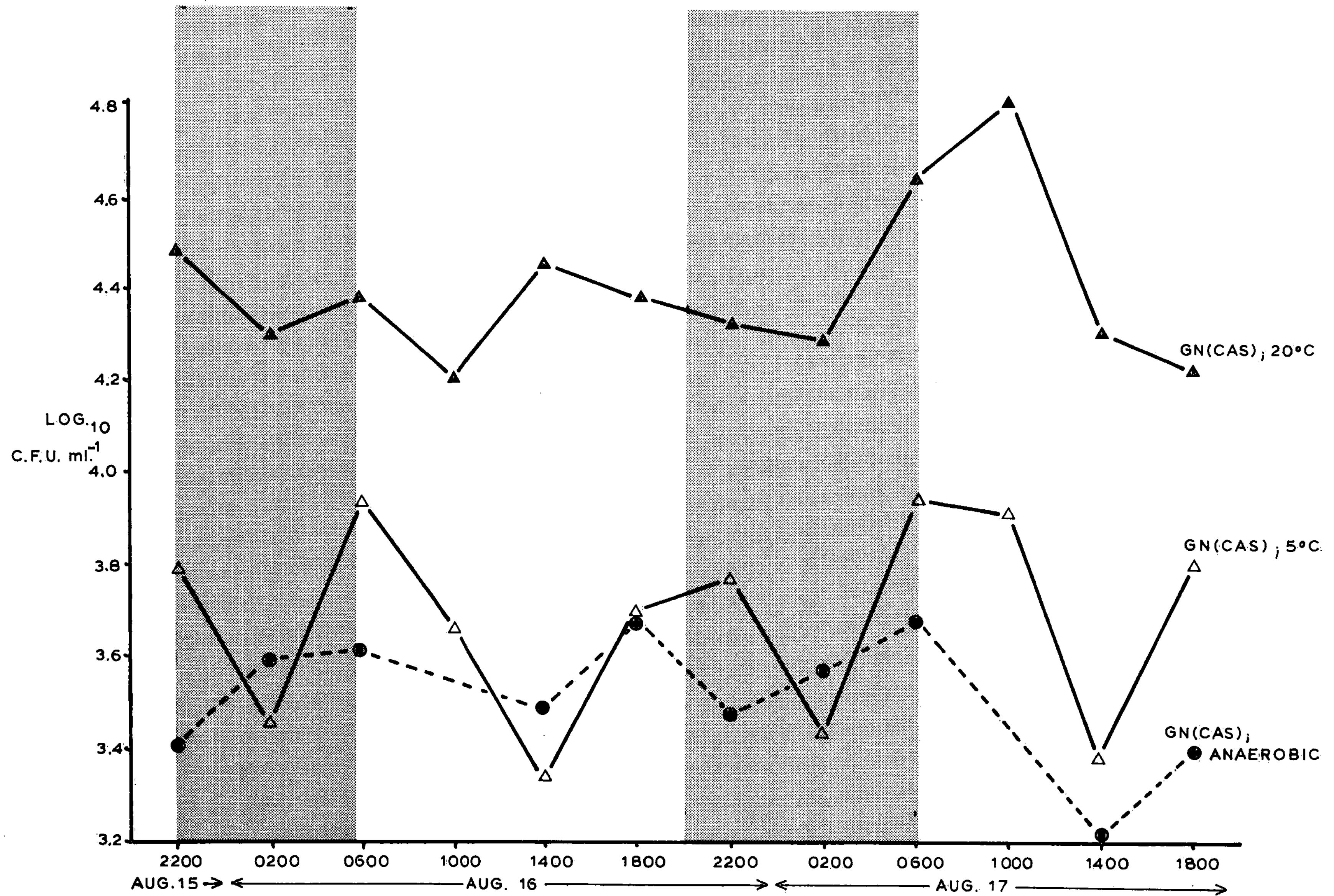


Figure 26. Fluctuations in sediment counts. The shaded area corresponds to the hours of darkness.

decreased during the night, whereas counts at 5°C increased slightly. This means that the estimate of percentage psychrophiles increased during the night when the water temperature dropped. If this is reliable, it suggests a far more rapid selection by temperature than suggested by Sieburth (1967). Viable counts in the sediment (Figure 26) showed a similar discrepancy between incubation temperatures. Counts at 5°C showed a 12-hour cycle peaking at dawn and dusk; 20°C counts showed a large increase at 6:00 - 10:00 a.m. hours on August 17. This corresponds to heavy rainfall experienced on the morning of August 17th amounting to 41 mm accumulation. The anaerobic counts decreased during the day when O_2 saturation was highest.

The mean viable counts over the 48-hour period (Table 25) showed that the sediment achieved counts one order of magnitude greater than the water. NO_3 and NH_4 assimilators comprised approximately 50% of the total heterotrophs. Psychrophiles comprised 17% of the population both in the sediment and water while anaerobes constitute 12% of the sediment total.

Table 25. Species composition in the water (No. of individuals in each species)

Species	Dark Period	Light Period
P. fluorescens I	58	58
P. cichorii	1	2
A. punctata	2	2
Acinetobacter spp.	14	6
Brevibacterium spp.	1	4
Total Species	5	5
Total Individuals	76	72
Diversity index (Simpson)	0.388	0.344
Diversity index (Shannon)	0.701	0.713

Table 26 shows the population obtained during this study. P. fluorescens I was dominant in the population (76% of the total), however, many facultative anaerobes and gliding bacteria observed in previous studies were not seen. The table does not show a diurnal trend in the population. Species composition in the sediment (Table 27) showed a light diurnal trend in the two major portions of the population; the obligate aerobes and the enterics. The obligate aerobes decreased in the light from 61% to 48% while the enterics increased from 29% to 37%. The high incidence of enterics supported the findings of Van Donsel & Geldreich (1971) that there is a high incidence of enterics in sediment.

Table 28 shows many differences in response frequency between sediment and water reported previously. The sediment bacteria exhibited a higher frequency of proteolysis, lipolysis, fermentation, gas production, and NO_3 reduction. The water bacteria demonstrated a higher incidence of ammonification, arginine dihydrolase, anaerobic growth, and substrate utilization. The table also permits differences between light and dark to be assessed. Thus proteolysis and lipolysis plus fluorescein production was higher in the light in both sediment and water. Glucose oxidation, fermentation and gas production in the sediment was higher in the light. PBH accumulation in the sediment was higher in the dark.

Table 26. Species composition in the sediment (No. of individuals in each species)

Species	Dark Period	Light Period
P. syringeae	28	27
P. alcaligenes	8	8
P. cepacia	15	2
Gram-negative rods *	2	3
C. violaceum	4	1
A. hydrophila hydrophila	3	7
K. pneumoniae	-	3
Enterobacter aerogenes	22	28
Enterobacter cloacae	3	-
Arthrobacter spp.	2	1
Corynebacterium spp.	-	3
Total Species	9	10
Total Individuals	87	83
Diversity index (Simpson's)	0.798	0.768
Diversity index (Shannon's)	1.736	1.668

* Further identification of oxidative rods not possible.

The factor analysis of the populations collected every 4 hours extracted a list of 12, mainly small, fragmentary factors. The eigenvalues for 10 of these factors were very small accounting for little variance. This reflected the large heterogeneity of the populations. Two factors however, were larger than most, together accounting for 40% of the variance elucidated by the total of 12 factors (Table 29). When graphed, these two factors showed a great dichotomy between sediment and river populations (Figure 27). The sediment samples all showed low nutritional versatility with high fermentation and proteolysis. The water populations were the converse. The correlations of Factor 3 in Table 30 suggest that when river counts are high, versatility is increased in the sediment. Similarly, when sediment counts are high, versatility is increased in the water. This implies some form of interface interaction. Factor 4 correlations showed that high sediment counts promoted low proteolysis and fermentation in both sediment and water. The significance of this is not known. Nitrate emerges as the major physico-chemical parameter affecting these two factors in the water.

Table 31 elucidated three forms of interactions. Interactions between the sediment and water were negligible. The organic N, C and P were tightly inter-related. This suggested that all three elements were possibly occurring in the same organic complex, perhaps allochthonous. The total heterotrophic counts are affected primarily by this organic trio of C, N, P. Vmax and the

Table 27. Variations in selected feature frequencies

Physiological Test	Sediment			Water		
	Dark	Light	Overall	Dark	Light	Overall
Casein hydrolysis	73	84	78	41	54	47
Gelatin hydrolysis	65	82	73	47	54	50
Tween 80 hydrolysis	71	83	77	50	60	55
Starch hydrolysis	28	29	29	22	25	23
Arginine dihydrolase	13	1	7	21	35	28
Fluorescein production	19	33	26	22	32	27
Phenazine production	8	32	20	14	8	11
Glucose oxidation	76	87	81	58	66	62
Glucose fermentation	76	84	79	48	45	46
Gas from fermentation	25	40	32	8	5	6
Gas from glucose/peptone	36	44	40	4	13	8
Ammonification	78	61	70	96	100	98
NO_3 reduction	79	79	79	57	67	62
Denitrification	2	1	1	2	6	4
Anaerobic growth	37	41	39	51	49	50
Levan formation	29	35	32	8	16	12
PBH accumulation	27	14	21	40	48	44
Gram stain	4	11	8	2	7	4
Motility	80	72	76	55	62	58
Urease	51	65	58	26	32	29
Lactose oxidation	6	6	6	17	10	13
Lactose fermentation	5	6	5	14	7	11
Methyl red	4	4	4	5	8	6
Voges Proskauer	26	38	32	17	11	14
5% salt tolerance	53	63	58	45	61	53
Substrate utilization	37	38	38	70	66	67
Total strains tested	106	103	208	107	102	209

Figures express the percentage positive in each category.

Table 28. The two major factors influencing the sediment and water population over a 48-hour period.

Factor #	Physiological Tests	Loadings	Eigenvalue	Interpretation
Factor 3	Growth on:-		0.0	Low nutritional versatility
	oxalate	-0.788		
	L-ornithine	-0.817		
	pelargonate	-0.603		
	pyruvate	-0.611		
	L-rhamnose	-0.792		
	sorbitol	-0.926		
	DL-serine	-0.772		
	D-tartarate	-0.735		
	L-tryptophan	-0.723		
	carboxymethylcellulose	-0.750		
	L-cystine	-0.656		
Factor 4	Glucose oxidation	-0.687	9.7	Lack of proteolysis and glucose catabolism, especially aerogenic fermentation.
	Glucose fermentation	-0.729		
	Gas from fermentation	-0.889		
	Gas from glucose/peptone	-0.843		
	Levan production	-0.839		
	Voges Proskauer	-0.893		
	Casein hydrolysis	-0.661		
	Gelatin hydrolsis	-0.639		

Table 29. Significant correlations of factor 3 and factor 4

	Sediment					Water	
	Turnover	pH	R(cas), 20°C	R(cas), 5°C	S(cas), 20°C	S(cas), 5°C	NO_3
<u>Factor 3</u>	0.569	0.641	-0.519	-0.719	-0.547	-0.566	-0.679
	(3.4%)	(1.2%)	(4.2%)	(0.4%)	(3.3%)	(2.7%)	(0.8%)
	S(cas), anaerobic				S(cas), 20°C	S(cas), 5°C	NO_3
<u>Factor 4</u>	0.764 (0.5%)				0.599 (2.0%)	0.706 (0.5%)	0.534 (3.7%)

R(cas) denotes viable counts on GN(cas) plates in the river water.

S(cas) denotes viable counts on GN(cas) plates in the sediment.

Table 30. Significant correlations between viable counts and the physico-chemical parameters

1) <u>Physico-chemical interactions</u>

	P total	N total	Organic C
P total		0.640 (1.7%)	0.899 (0%)
N total			.818 (0.1%)
Organic C			

2. <u>Physico-chemical/water interaction</u>

	Organic C	Total P	Total N	pH	O_2 Saturation	Vmax	Temp. °C
GN(cas); 20°C	0.870 (0%)	0.768 (0.2%)	0.895 (0%)			0.749 (0.4%)	
GN(cas); 5°C				-0.725 (0.4%)			
% psychrophiles	-0.532 (3.7%)		-0.570 (3.4%)		-0.777 (0.2%)		
Turnover				0.658 (1.4%)			-0.589 (2.8%)
Vmax	0.758 (0.3%)	0.777 (0.2%)	0.658 (1.9%)				

3. <u>Physico-chemical/sediment interactions</u>

	O_2 Saturation	Chlorophyll a	NO_3
GN(cas); 20°C	-0.449 (8.3%)		0.582 (2.4%)
GN(cas); 5°C		-0.440 (7.6%)	
GN(cas); anaerobic		-0.757 (0.6%)	

Table 31. Mean viable counts

Media	Meduxnekeag		Dunbar	
	Sediment	River	Sediment	River
Glucose nitrogen(GN) + casamino acids(cas)	$3.04 \times 10^5 \pm 3.87 \times 10^5$	$9.68 \times 10^3 \pm 1.20 \times 10^4$	$8.51 \times 10^4 \pm 1.32 \times 10^5$	$6.20 \times 10^3 \pm 8.98 \times 10^3$
Glucose nitrogen(GN) + nitrate(NO_3)	$1.43 \times 10^5 \pm 2.92 \times 10^5$	$4.77 \times 10^3 \pm 6.14 \times 10^3$	$3.45 \times 10^4 \pm 5.39 \times 10^4$	$1.86 \times 10^3 \pm 2.18 \times 10^3$
Glucose nitrogen(GN) + ammonia(NH_4)	$1.45 \times 10^5 \pm 1.98 \times 10^5$	$3.43 \times 10^3 \pm 3.46 \times 10^3$	$4.73 \times 10^4 \pm 7.82 \times 10^4$	$6.91 \times 10^3 \pm 1.25 \times 10^4$
Nitrogen fixation medium (N20)	$1.91 \times 10^5 \pm 4.88 \times 10^5$	$2.38 \times 10^3 \pm 1.23 \times 10^4$	$1.40 \times 10^4 \pm 2.10 \times 10^4$	$3.25 \times 10^2 \pm 3.41 \times 10^2$
Nitrogen fixation medium (N21)	$1.26 \times 10^4 \pm 2.05 \times 10^4$	$3.16 \times 10^3 \pm 6.30 \times 10^3$	$1.01 \times 10^4 \pm 1.84 \times 10^4$	$8.20 \times 10^2 \pm 1.17 \times 10^7$
Nitrogen fixation medium (N22)	$1.78 \times 10^5 \pm 1.96 \times 10^5$	$2.93 \times 10^4 \pm 3.69 \times 10^4$	$2.08 \times 10^5 \pm 3.39 \times 10^5$	$4.58 \times 10^3 \pm 5.77 \times 10^3$
NO_3 red$^{\underline{n}}$	$8.48 \times 10^4 \pm 8.08 \times 10^4$	$2.38 \times 10^3 \pm 2.70 \times 10^3$	$6.83 \times 10^3 \pm 7.31 \times 10^3$	$4.46 \times 10^2 \pm 5.00 \times 10^2$

Media designations explained in the methods. Means given $\pm$ one standard deviation.

Sediment counts given per gram wet weight; water counts per ml.

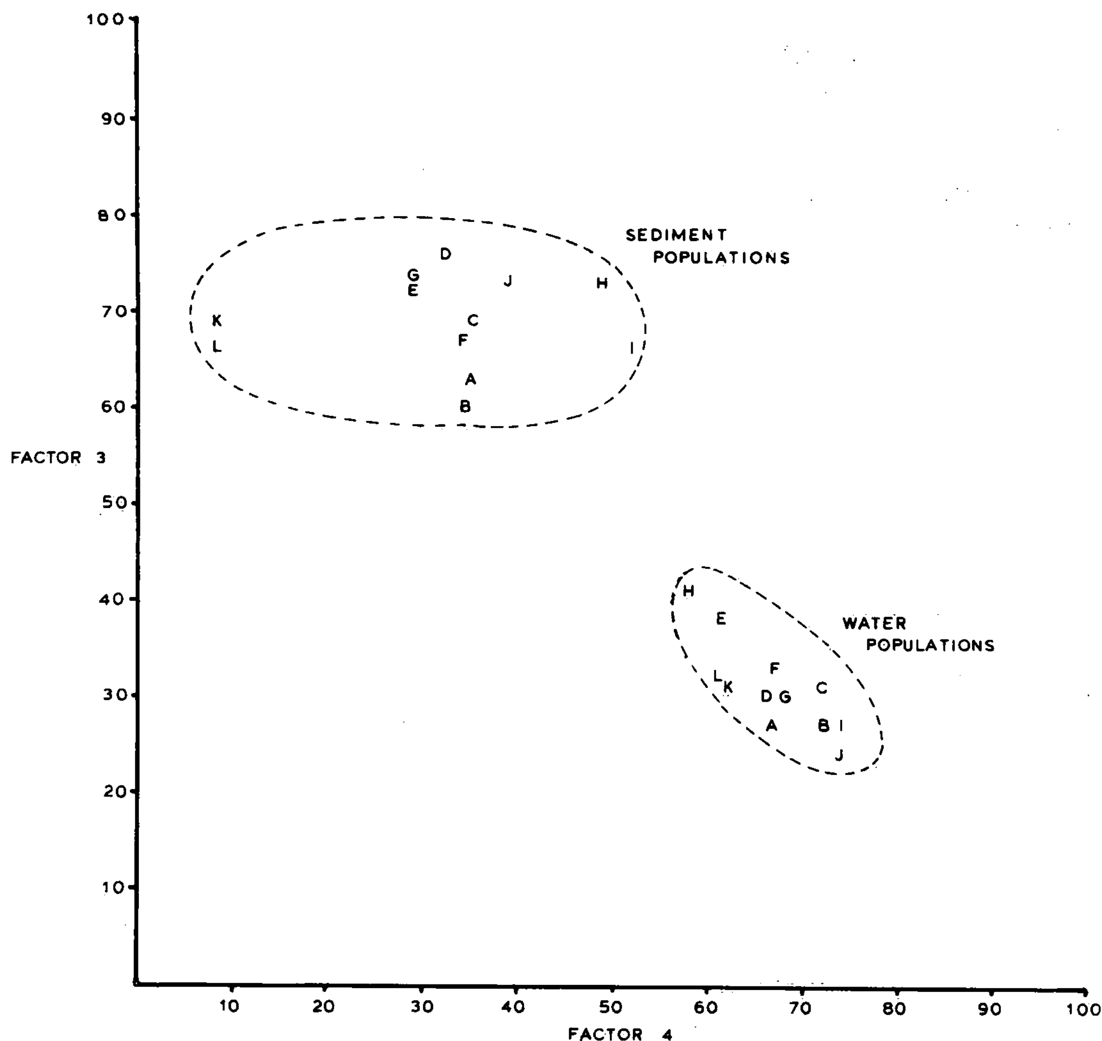


Figure 27. Projections of Factors 3 and 4 on sediment and water samples. Sample A corresponds to 10:00 p.m. on 15th August. The sample letters then run alphabetically every 4 hours to sample L at 6:00 p.m. on 17th August.

total heterotrophic count at 20°C is correlated with the organics. Albright & Wentworth (1973) found identical correlations when they proposed Vmax as a kinetic estimator of eutrophication. The correlations of sediment counts with O_2 saturation, chlorophyll a and NO_3 are intriguing because these three variables are intimately involved with primary production. The connection between sediment bacteria and primary production in the water can only be speculated upon.

Diurnal variation in the biological, chemical and physical parameters was observed in this study. Certain predictable patterns were detected. The % saturation of O_2, pH, organic carbon and total phosphorus was reduced at night. This reduction was accompanied by a decrease in chlorophyll a and mesophile bacterial counts in water and sediment samples. These reflect a general reduction of the biosynthetic activities resulting in the build-up of biomass. Certain activities, such as the oxidation of nitrogen and an increase in turnover time, were stimulated by the arrival of darkness. If the first figure of the Vmax is disregarded, the peaks were reached within three hours after maximum turnover time was recorded. A distinctive 12-hour diurnal variation was observed in psychrophile sediment counts, peaks being reached at dawn and dusk. The anaerobic counts in the sediment decreased when the O_2 saturation was highest.

The bacterial population showed some response to the sunlight, with enterics increasing with temperature and obligate aerobes decreasing in response to the drop in O_2. Some diurnal variation in species was observed in these samples. It should be noted that this feature was observed in July as reported by Cormier in this report, i.e. a slight diurnal variation in the predominant species.

The factor analysis clearly underscores the very great difference between these two populations, i.e. that each econiche is a separate and distinct entity. Factor 3, turnover, is negatively correlated with bacterial counts and nitrate, underlining the increase in turnover when a drop in biomass activity was observed. Factor 4, anaerobic counts, is supported as expected by sediment counts and shows a positive correlation to nitrate. Attempts to link activities in the sediment and water using correlation have shown some rather tenuous interface activity. A larger body of data with greater variation is necessary to effectively utilize factor analytical methods.

Interactions of Heterotrophic Bacteria in the Sediment and Water Phases of Two Contrasting Rivers

C.R. Bell, M.A. Holder-Franklin and M. Franklin

SUMMARY

The Meduxnekeag and Dunbar rivers, in addition to having differing water uses and physico-chemical parameters, show large differences in sediment structure. The Meduxnekeag has a dark grey silty mud, whereas the Dunbar has a coarse gravel.

The sediment counts are always at least one order of magnitude greater than the river counts. Counts in the Meduxnekeag sediment are about ten times higher than counts in the Dunbar sediment. Correlations between total heterotrophic counts in sediment and water are insignificant. There is thus little overall interchange in numbers.

The Meduxnekeag sediment is highly atypical of these river systems in lacking fluorescent pseudomonads. The Dunbar sediment harbours twice as many obligate aerobes as does the overlying water.

The sediment populations in both rivers accumulate more PBH than the water populations, have a lower frequency of anaerobiosis and fermentation, are more mesophilic and less proteolytic and lipolytic.

Four factors express the variability in the Meduxnekeag populations. Only three factors emerge for the Dunbar populations. These three factors all have a strong oxidative bias. Oxidative bacteria are believed to reside in the Dunbar sediment and possibly seed the overlying water. These populations are more affected by changes in water parameters than the water populations.

Fermentative bacteria are believed to reside in the Meduxnekeag sediment and seed the overlying water. The Meduxnekeag sediment populations are largely unaffected by changes in the physico-chemical nature of the water. Viable counts in the Meduxnekeag water are influenced primarily by rainfall and organic C. Sediment populations are only effected by NH_4. Ammonia is the key variable influencing both sediment and water counts in the Dunbar. These differences between sediment populations are attributable largely to the difference in sediment structure.

INTRODUCTION

An aquatic ecosystem, in addition to the many depth-related niches, also has a unique area of interaction with the sediment. We assume that in shallow rivers and streams, this boundary of land and water must play a more influential role in the milieu of the water column above it.

Many of the nutrient transformations in aquatic habitats are known to occur in the sediment and be mediated through microorganisms (Kuznetsov, 1968). The sediment can act either as a source or sink for nutrients and profoundly affect the quality of the overlying water (Foess & Feng, 1971). The role of the sediment is particularly pertinent in the cycling of nitrogen (Brezonik, 1972) where much attention has been focused on denitrification as a nitrogen sink (Keeney, 1973; Terry & Nelson, 1975).

Bacteria also exert more diverse influences in the sediment. Dugan et al. (1970) found that agglutination and precipitation of microparticles by the polymer fibrils of bacteria play a role in sediment formation. Burns & Ross (1972) found that bacterial oxidation of organics accounted for 88% of the oxygen depletion at the Lake Erie sediment-water interface. Most of the bacteria encountered are active in other transformations (e.g. proteolysis and ammonification) (Bandurski, 1965) and are very comparable to the genera found in soils (Kuznetsov, 1975).

The labile organic carbon pool in the sediment is suspected of being much higher than the pool in the water column (Wood, 1970; Brinkhurst et al. 1971). The distribution and role of heterotrophic bacteria in such organically rich layers is now receiving attention (Dutka et al. 1974).

The heterotrophic bacterial populations in the water of two tributaries of the Saint John River have been under investigation in this laboratory for several years (Bell, Holder-Franklin & Franklin, 1980). The two rivers studied, the Meduxnekeag and the Dunbar, are both considered oligotrophic, but the former does receive domestic and industrial effluent, whereas the Dunbar is a small, pristine woodland river (Plate 1). This paper examines the heterotrophic bacteria in the sediment of these two rivers and explores interactions across the sediment-water interface.

MATERIALS AND METHODS

Shallow water sediment cores were obtained with a sampler designed by Daniel & Chesters (1971). River water samples were taken every alternate week from each river between February 1977 to February 1978 as a part of an ongoing programme. Sediment samples were taken from the Dunbar and seven such samples from the Meduxnekeag River. Viable count estimates were performed on these samples on the basis of 1 gram wet

weight as a slurry. Estimates of total heterotrophs, NO_3-assimilators and NH_4-assimilators were achieved using a defined glucose-nitrogen medium (Brown & Stanley, 1972) with a supplement of 0.2% w/v casamino acids, GN(cas), 200 g $N\text{-}NO_3ml^{-1}$, $GN(NO_3)$ and 200 g $N\text{-}NH_4ml^{-1}$, $GN(NH_4)$ respectively. Putative nitrogen fixers were estimated on the media of Norris (1959), (N20); Maruyama et al. (1974), (N22) and Kawai & Sugahara (1971), (N21). Denitrifiers and nitrate reducers were estimates on the medium of Sreenivasan & Venkataraman (1956), (D30), incubated in a Gaspak jar. Total heterotroph counts were performed by the spread plate technique, all other estimates were as Most Probable Number's (M.P.N.). All incubations were performed at 20°C.

Colonies obtained on the casamino-acid agar plate from samples taken on 12th September, 26th September and 17th October from the Dunbar River and on 25th July, 19th September and 3rd October from the Meduxnekeag River were picked off at random and purified. Approximately 100 isolates were obtained from each river. These sediment isolates were then examined together with river isolates obtained from identical incubations at 20°C by numerical taxonomy. Precise details of the tests employed and methodology are available elsewhere (Bell, Holder-Franklin & Franklin, 1980) and a modification of the methods described in this report. A factor analysis was performed on the populations isolated from each river using the Varimax method of rotation (Holder-Franklin 1980). The positive test response frequencies were used as the subject scores in this analysis. Correlations between the factor projections for each sample date and a range of 24 physico-chemical parameters were calculated with the aid of the Statistical Package for the Social Sciences (SPSS). Assay methods for the physico-chemical parameters are explained in Bell, Holder-Franklin & Franklin (1980) and Cormier (1978).

RESULTS AND DISCUSSION

The structure of the sediment at the Dunbar sample site is of a coarse gravelly nature, whereas the Meduxnekeag sediment is a dark grey to black silty mud. Table 32 shows that the Meduxnekeag mud consistently produces higher viable counts than the Dunbar sediment. Vanderpost & Dutka (1971), in a more exhaustive survey, also found that bacterial density was inversely related to sediment particle size. The counts obtained on the inorganic nitrogen media, $GN(NO_3)$ and $GN(NH_4)$, constitute about 40-50% of those obtained on the organic source, suggesting that approximately half of the population may be incapable of assimilating inorganic nitrogen. An exception is in the Dunbar River where NH_4 assimilators form the largest count. Nitrate reducers account for a much larger proportion of the population in the Meduxnekeag (25-30%) than in the Dunbar (7-8%). This would be expected from the anoxic appearance of the Meduxnekeag sediment and the much lower O_2 saturation values in the river water throughout the year (Bell, Holder-Franklin & Franklin, 1980). The medium of Maruyama et al. (1970), designated N22, produced consistently high counts, sometimes higher than all other media (Table 31). Although designed to estimate nitrogen-fixing bacteria, the media does include 0.05 g yeast extract per litre. Medium composed with low organic concentrations may prove more applicable in these low nutrient rivers.

Correlations between the viable counts in the sediment and water phases in the Dunbar River (Table 32) are insignificant apart from two low nutrient media (N20 and N22). The Dunbar River was shallow (approx. 40 cm deep) and turbulent (mean summer flow rate was 70 cm/sec.), therefore, bacteria may benefit from some adherence mechanism. The morphometric parameters of the Meduxnekeag differ: appro . depth 110 cm, mean summer flow rate = 30 cm^3/sec. The correlation between NO_3 reducers was expected for reasons just stated. Ammonium ions are absorbed very quickly by silty sediments, so the correlation between NH_4 assimilators may represent seeding from the sediment to the water. However, the correlations between the low nutrient media, N21 and N22, were anomalous.

Table 33 shows the bacterial isolates which clustered in the numerical taxonomy and were identified. The Meduxnekeag sediment appeared to possess a very heterogenous population; 105 strains were tested but only 30 of these showed enough homology to cluster (29%). The remaining three categories had clustering percentages from 67% to 84%. Pseudomonas fluorescens I dominated the population in these two rivers throughout the year of study (Bell, Holder-Franklin & Franklin, 1980). However, previous studies by Holder-Franklin et al. (1978) indicated that this predominance varied with the environment.

The bacterial populations in the sediment, when expressed as a percentage of the total (Table 34), demonstrated that both sediments showed an increase in the Pseudomonas accumulating PBH compared to the water. Indeed P. palleronii, P. delafieldii and P. acidovorans, all PBH accumulators, occur exclusively in the sediment. The Meduxnekeag sediment shows a drastic reduction in fluorescent aerobes with a significant increase in facultative anaerobes. The Dunbar sediment, in contrast, is almost exclusively aerobic with minimal occurrence of gliding bacteria. The Dunbar River exhibits higher proportions of facultative anaerobes and gliding bacteria, Cytophaga fermentans occurring exclusively in the water.

When all isolates are considered, both unclustered and clustered, a similar picture emerges (Table 35). PBH accumulation is again greater in the sediment. The advantage of storing these fat granules when in the sediment is obscure. Ammonification is extremely high in all cases. Byrnes et al. (1972) have shown that sediment NH_4 rapidly reaches equilibrium with the overlying water and that much of the NH_4 released through ammonification is returned to the overlying water. This may occur in the slightly O_2 undersaturated waters of the Meduxnekeag, but in the O_2 rich waters of the Dunbar it is likely that the NH_4 would rapidly be nitrified (Chen et al. 1972; Tuffey et al. 1974). Vanderborght & Billen (1975) observed nitrification in the upper few centimetres of sandy sediments but not in muddy sediments. The appreciable phosphatase activity in both sediments suggests turnover of phosphate from the sediments. Ayyakkannu & Chandramohan (1971) noted a relationship between phosphate content, the numbers of phosphatase solubilizing bacteria and phosphatase activity

Table 32. Correlations among viable counts between the sediment and water phases.

Water media	vs. Sediment media	Correlation coefficients Meduxnekeag	Dunbar
GN (cas)	GN (cas)	n.s.	n.s.
GN (NO_3)	GN (NO_3)	n.s.	n.s.
GN (NH_4)	GN (NH_4)	0.766 (2.2%)*	n.s.
N20	N20	n.s.	0.607 (4.1%)
N21	N21	0.993 (0.1%)	n.s.
N22	N22	0.690 (6.5%)	0.769 (1.3%)
D30	D30	0.631 (6.4%)	n.s.

Media designations are explained in the methods
* (Significance level)
n.s. denotes not significant; 10% significance

Table 33. Variations in bacterial species (No. of individuals in each species)

Species	Meduxnekeag Sediment	Meduxnekeag River	Dunbar Sediment	Dunbar River
P. fluorescens I	2	53	40	33
P. putida	2	1	7	3
P. alcaligenes	6	-	-	-
A. eutrophus *	-	2	4	3
P. mallei	-	1	4	-
P. cepacia	4	-	-	1
P. solanacearum	2	5	2	2
P. palleronii	-	-	4	-
P. delafieldii	-	-	4	-
P. acidovorans	-	-	5	-
A. hydrophila hydrophila	4	1	2	8
A. punctata	7	4	-	-
F. devorans	-	8	7	7
Flexibacter succinicans	3	10	1	9
Cytophyga fermentans fermentans	-	-	-	16
Citrobacter intermedius	-	-	2	5
Enterobacter aerogenes	-	5	-	-
Total individuals	30	90	82	87
Total species	8	10	12	10
Diversity Index (Simpson):	0.876	0.631	0.741	0.800
Diversity Index (Shannon):	1.854	1.415	1.789	1.819

*Alcaligenes

Table 34. Variations in the percentage of bacterial types

Type	Meduxnekeag		Dunbar	
	Sediment	River	Sediment	River
Obligate aerobes (fluorescent; do not accumulate PBH)	33%	62%	62%	45%
Obligate aerobes (accumulate PBH)	20%	7%	23%	3%
(Total obligate aerobes)	(53%)	(69%)	(85%)	(48%)
Facultative anaerobes	37%	15%	11%	17%
Gliding bacteria	10%	11%	1%	29%
Enteric bacteria	-	5%	3%	6%
Total individuals	30	90	82	87

Table 35. Selected feature frequencies of the population from the sediment and water phases

Physiological Test	Meduxnekeag		Dunbar	
	Sediment	River	Sediment	River
Overall substrate utilization	39	47	51	45
Inorganic N-assimilation	98	96	99	99
Putative N_2-fixation	96	93	100	98
Growth on carboxymethylcellulose	15	4	1	14
Starch hydrolysis	42	31	14	43
Casein hydrolysis	65	79	64	81
Gelatin hydrolysis	69	78	62	76
Tween 80 hydrolysis	47	65	57	66
Production of fluorescein	6	42	49	35
Production of phenazine	17	10	10	23
Growth at 4°C	51	90	79	90
Growth at 37°C	57	50	57	35
Accumulation of PBH	63	25	47	22
Fermentation of glucose	64	67	28	52
Gas from glucose	36	56	39	47
Anaerobic growth	29	50	9	14
Phosphatase	44	44	37	49
Ammonification	91	96	94	97
Denitrification	1	3	8	2
Total strains tested	105	135	98	133

Figures express the percentage positive in each category.

in sediments. Denitrification, however, is extremely low, so little nitrogen is probably lost as gaseous N to the atmosphere. Anaerobic trends and fermentation tend to be lower in the sediment. This is understandable in the Dunbar but in the Meduxnekeag mud such results are curious.

The sediment does possess a bacterial population with different temperature characteristics from the water bacteria. The sediment bacteria tend to be more mesophilic showing more growth at 37°C and less at 4°C. Bell & Dutka (1972) also found little evidence for truly psychrophilic bacteria in Lake Ontario sediments. The production of hydrolytic exoenzymes tends to be greater in the water than in the sediment with the exception of starch hydrolysis. Starch hydrolysis is higher in the Meduxnekeag sediment but lower in the Dunbar sediment. Starch is an important organic input in both these rivers and starch hydrolysis on a seasonal basis was found to have a strong correlation to oxygen content (Bell, Holder-Franklin & Franklin, 1980). In the Meduxnekeag, however, starch hydrolysis was greatest at low O_2 saturation, while in the Dunbar it was highest at high dissolved O_2 concentrations. These observations would explain the discrepancy between starch hydrolysis in the sediments in the two rivers. A similar discrepancy occurs in percentage overall substrate utilization between the two rivers. It is highest in the Meduxnekeag River and in the Dunbar sediment. The in situ aerobic decomposition rate would probably be reduced in the fine silt of the Meduxnekeag (Hargrave, 1972). This restraint may influence the ability of sediment isolates to degrade organic compounds in the laboratory. The utilization of glucose, as shown by studies with radioactive tracers, has always been found to be faster in the sediment (Harrison et al. 1971; Chochair & Albright, 1978). Whether this finding extends to other organics is unknown.

The factors extracted by the factor analysis (Table 36 and 37) reflect many of the differences already discussed in Table 28. These factors show much similarity to previous factors extracted from a more exhaustive study on the variations in the bacterial populations (Holder-Franklin et al. 1978). Interactions between the

Table 36. Major factors influencing the Meduxnekeag river/sediment populations

Factor #	Physiological test	Loadings	Eigenvalue	Interpretation
Factor 1	PBH accumulation	-0.943	18.4	Psychrophilic, fluorescent, oxidisers not accumulating PBH nor reducing NO_3
	Oxidase reaction	-0.718		
	NO_3 reduction	-0.898		
	Starch hydrolysis	-0.603		
	Fluorescein production	0.960		
	Arginine dihydrolase	0.983		
	Glucose oxidation	0.759		
	Growth at 4°C	0.860		
	Ammonification	0.823		
Factor 2	Lactose fermentation	0.942	11.4	Fermentation and salt tolerance
	Glucose fermentation	0.839		
	5.0% salt tolerance	0.777		
Factor 3	N-NO_3 assimilation	-0.935	12.8	Aerogenic anaerobes not assimilating N-NO_3 or scavenging N_2
	N-NH_4 assimilation	0.741		
	Growth on N_2-free media: N20*	-0.934		
	N21	-0.600		
	Anaerobic growth	0.782		
	Gas from glucose/peptone	0.753		
	Gas from glucose/fermentation	0.844		
Factor 4	Casein hydrolysis	-0.882	13.4	Inability to produce exo-enzymes and degrade complex polymers
	Gelatin hydrolysis	-0.852		
	Starch hydrolysis	-0.564		
	Urease	-0.689		
	Phosphatase	-0.763		
	Growth at 37°C	-0.880		

* N20 = media of Norris (1959)

N21 = media of Kawai and Sugahara (1971)

Table 37. Major factors influencing the Dunbar river/sediment populations

Factor #	Physiological test	Loadings	Eigenvalue	Interpretation
Factor 1	Starch hydrolysis	0.913	19.1	Starch hydrolysis with lack of fluorescent oxidative trends and no accumulation of PBH
	Growth on carboxymethyl-cellulose	0.940		
	Fluorescein production	-0.762		
	Arginine dihydrolase	-0.627		
	Methyl red reaction	-0.978		
	Growth at 37°C	-0.522		
	Oxidase reaction	-0.840		
	Glucose fermentation	0.758		
	PBH accumulation	-0.657		
Factor 2	Casein hydrolysis	0.972	15.0	Psychrophilic lipolysis and proteolysis with associated oxidative trends
	Gelatin hydrolysis	0.988		
	Tween 80 hydrolysis	0.992		
	Growth at 4°C	0.957		
	Arginine dihydrolase	0.538		
	Fluorescein production	0.512		
	Glucose oxidation	0.688		
	Glucose fermentation	-0.834		
Factor 3	Motility	0.773	12.6	Mesophiles unable to ammonify or catabolize simple sugars
	Growth at 37°C	0.785		
	Ammonification	-0.992		
	Glucose oxidation	-0.513		
	Lactose oxidation	-0.892		
	Glucose fermentation	-0.630		
	Gas from glucose	-0.912		
	Anaerobic growth	-0.785		

Table 38. Significant correlations between Meduxnekeag factor scores and physico-chemical parameters

	Sediment			River		
Factor 1		Na -0.995 (3.0%)*		Chlorophyll *a* -0.994 (3.4%)		
Factor 3	Turbidity -0.997 (2.5%)	Temp. 0.996 (2.7%)	SO_4 0.989 (4.8%)	NH_4 0.994 (3.4%)	NO_3 0.989 (4.8%)	Sun 0.999 (1.5%)
Factor 4	Total P -0.993 (3.8%)	Turbidity -0.996 (2.9%)				

* (Significance level)

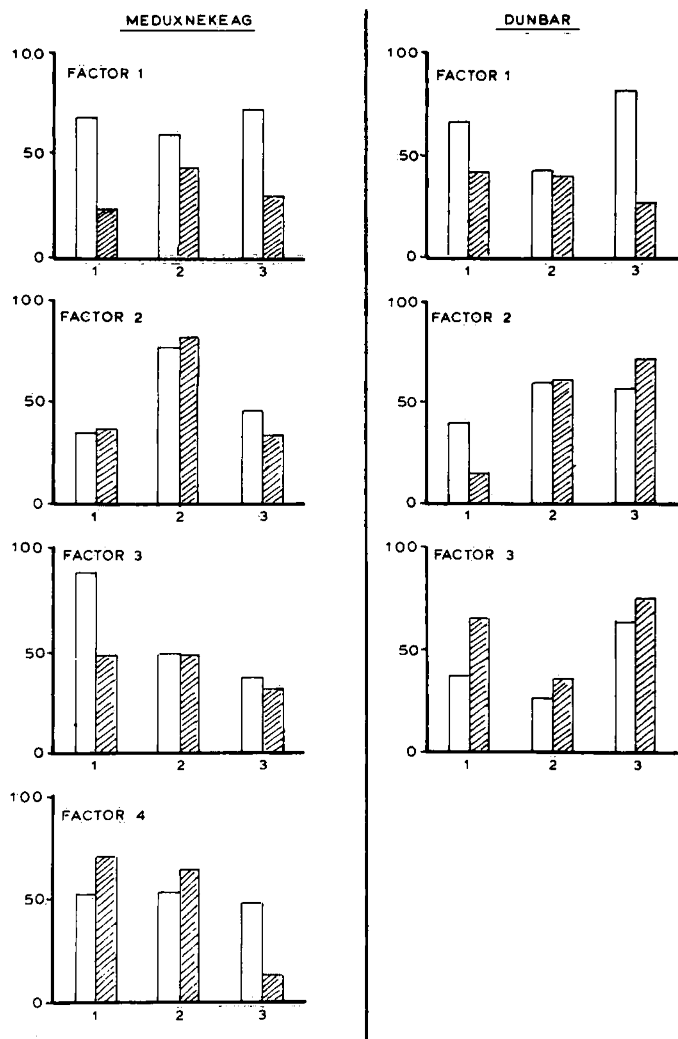


Figure 28. Factor projections per river for each of three sample dates. Meduxnekeag dates 1, 2 and 3 correspond to 25th July, 19th September and 3rd October respectively. Dunbar dates 1, 2 and 3 correspond to 12th September, 26th September and 17th October respectively. Hatched bars represent the sediment populations, clear bars the water populations. Factor numbers as described in Tables 6 and 7.

factor projections for a given sample date can be better visualized when the projects are graphed (Figure 22). Projections are transformed to a mean of 50 and a standard deviation of 20.

In the Meduxnekeag, factor 1 was always higher in the water than the sediment, showing the scarcity of fluorescent oxidizers in the sediment. Factor 2 demonstrates a close correspondence between sediment and water projections on each sample date. Factor 2 represents fermentative metabolism. The projections for factor 1 have already shown the preponderance of fluorescent oxidizers in the water, so it seems unlikely that the water could affect the fermentative capacity of the sediment. The similarity in factor 2 loadings is believed to represent the seeding of the water column by fermentative bacteria indigenous to the sediment. The projections for factor 2 in the river populations also show good correlation to factor 1 projections in the sediment. As these factor 1 projections are all below the mean of 50, the inverse interpretation of the factor applies, viz, relative lack of fluorescent oxidizers, accumulating PBH and reducing NO_3. The prevalence of these bacterial types further endorses the hypothesis that the fermenters in the river water are originating from the sediment. Factor 3 projections also show some similarity between water and sediment populations. The projections decrease across the three sample dates with the sediment and water projections corresponding in height. Part of this factor represents aerogenic anaerobiosis and complements the hypothesis stated above. The projections for factor 4 are greater in the sediment on dates 1 and 2. This infers that the hydrolyzing enzymes reflected in factor 4 were low in the sediment on these dates. This result has been observed earlier. The reversal on date 3 is anomalous.

In the Dunbar populations, factor 1 is again consistently higher in the water phase. This represents the proliferation of fluorescent, PBH accumulating, pseudomonads in the sediment. Factor 2 projections increase across the sample dates. These dates range from the 12th of September to the 17th October and by the last date the factor has become more important in the sediment than the water. Because this factor has a psychrophilic component to it this trend may reflect a seasonal shift of lipolysis and proteolysis to the sediment during the winter. Seasonal data does show a reduction in these activities in the water under the ice cover (Bell, Holder-Franklin & Franklin, 1980). Factor 3 again shows a correspondence in height between the sediment and river projection on each date. The sediment projection for factor 3 also correlates closely with the river projections for factor 1. This infers that when the incidence of mesophiles unable to ammonify or catabolize simple sugars (factor 3) is high in the sediment, the lack of fluorescent oxidizers with starch hydrolysis (factor 1) will be high in the water. Apart from starch hydrolysis these two factors almost measure the same characteristic. This shows the strong influence of psychrophilic, fluorescent and oxidative trends in the Dunbar and suggests a strong homogeneity between sediment and river.

The correlations between factor scores and physico-chemical parameters (Tables 38 and 39), although only based on 3 sample points, do show some strong correlations. Correlations in the Meduxnekeag occur largely in the river; the sediment is relatively unaffected. This is particularly pertinent for factor 1, as the sediment has been shown to be almost devoid of fluorescent oxidizers. Unfortunately, interpretations of the correlations in the river with Na and chlorophyll a are not apparent. In the seasonal study it was suggested that many of the fluorescent oxidizers were washed in with rainfall. Perhaps these correlations reflect some similar influence. Factor 2 shows no correlations. This is in keeping with the suggestion that the fermentative portion of the population resides in the sediment. Factor 3 was also stated to reflect part of this sediment influence. The negative correlation with turbidity could represent disturbance of the sediment and loss of anaerobes to the water phase.

Table 39. Significant correlations between Dunbar factor scores and physico-chemical parameters

	Sediment				River		
Factor 1	Color -0.995 (3.3%)*	Ca 0.996 (2.8%)	Na 0.999 (0.6%)	Total P -0.996 (2.8%)			
	Rain -0.994 (3.4%)	Organic C -0.996 (2.8%)	Inorganic C 0.999 (1.2%)				
Factor 2	Sun -0.998 (1.9%)				NH_4 -0.988 (4.9%)		
Factor 3					S.C. -0.998 (2.0%)	Mg -0.999 (1.3%)	Chlorophyll a -0.993 (3.6%)

S.C. = Specific Conductance

* (Significance level)

Table 40. Correlations between viable counts and physico-chemical parameters

Meduxnekeag River

Sat^n	Specific Conductance	Colour	Alkalinity	NH_4		Organic C	Inorganic C	Diss. C	O_2	% O_2 Rain
Water counts	-0.336 (8%)	0.797 (0.1%)	-0.377 (6%)	n.s.	-0.50 (1.5%)	0.759 (0.1%)	-.335 (9%)	-0.436 (3.1%)	0.713 (0.1%)	-0.407 (4.2%)
Sediment counts	n.s.	n.s.	n.s.	0.852 (0.7%)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Dunbar River

	NH_4	Organic C	Sun
Water counts	0.419 (2.6%)	n.s.	-0.439 (2.1%)
Sediment counts	0.557 (6%)	-0.553 (6.1%)	n.s.

* (Significance level)

n.s. denotes not significant; 10% significance

The inclusion of hours of sunshine as a correlating variable is interesting in that in an earlier paper, sunshine was shown to be negatively correlated to numbers of P. fluorescens I (Bell, Holder-Franklin & Franklin, 1980). This may reflect a competitive process. NO_3 assimilation and N_2 scavenging were also shown to decrease in the Meduxnekeag in winter (Bell, Holder-Franklin & Franklin, 1980). The decrease in factor 3 with decreasing temperature endorses this. Phosphatase is one of the tests included in factor 4. The negative correlation of total P with factor 4 suggests that the more total P there is, the more phosphatase in the sediment. Phosphate is known to inhibit phosphatase production, so that a causal interpretation of total P on phosphatase is invalid. It seems more plausible to suggest that increased phosphatase activity in the sediment is elevating the concentration of total P in the water.

In the Dunbar, the situation is reversed, with more correlations occurring in the sediment than in the water. The omission of any correlations with factor 1 in the river suggests that fluorescent oxidizers may reside mainly in the sediment. Starch hydrolysis is seen to decrease in the sediment with increasing concentrations of organic C and total P. These conditions presumably favour the growth of the fluorescent oxidizers. The emergence of sun and NH_4 as correlating variables with factor 2 also occurred in the seasonal study. However, the germicidal effect of light is unlikely to penetrate the sediment, so that any correlation with sunshine has to be explained by phototaxis, about which little is known in the natural environment. Ammonia is believed to be the limiting nutrient in this river. Previous results showed that increasing NH_4 concentrations increased the diversity of the population. Available NH_4 appears to encourage other species to flourish at the expense of the fluorescent pseudomonads and hence produce the negative correlation with factor 2. The negative correlation of chlorophyll a, Mg and specific conductance with factor 3 is similar to that observed with factor 1 in the Meduxnekeag. Its significance is not known.

Table 40, the correlations between viable counts and physico-chemical parameters, shows identical results to those obtained in the larger study (Bell, Holder-Franklin & Franklin, 1980). The heterotrophic counts in the Meduxnekeag water are influenced primarily by rain and organic C. Rainfall has been cited as influencing bacterial populations (Collins, 1960, 1970; Chen, 1968; Guthrie, 1968). The effects are subtle however, often mediated through other agencies such as shown here with specific conductance and alkalinity. The negative correlations with oxygen, both as dissolved concentration and percentage saturation, are believed to represent O_2 depletion through heterotrophic respiration. The strong correlation of sediment counts to NH_4 is believed to reflect the rapid adsorption of ammonia to sediment particles.

The same influence of ammonia is seen in the Dunbar, both on the sediment and water counts. This is due to its limiting effect on the diversity of the population. The negative correlation to sunlight may be due to the germicidal effect of light. Harmful effects of sunlight on bacterial suspensions have been documented (Jagger, 1975; Krinsky, 1976; Fedorak & Westlake, 1978). However, the emergence of sunlight as a variable affecting the sediment makes the necessary penetration of light a problem. These phenomena may have to be explained on the basis of phototaxis.

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Reference Strains and Source

Reference Strain	Reference Collection and Strain Number
Pseudomonas chlororaphis	ATCC 9446
Pseudomonas aureofaciens	13985
Pseudomonas fluorescens Biotype G	17386
Pseudomonas aeruginosa	15442
Pseudomonas aeruginosa	14216
Pseudomonas fluorescens Biotype III	17400
Pseudomonas fluorescens Biotype I	13525-1
Pseudomonas fluorescens Biotype I	13525-2
Pseudomonas fluorescens Biotype II	17482
Pseudomonas fluorescens Biotype II	17816
Pseudomonas putida	12633
Rhizobium leguminosarum	10314
Pseudomonas cepacia	25416
Pseudomonas marginata	10248
Pseudomonas fragi	4973
Pseudomonas denitrificans	13867
Zoogloea ramigera	19544
Pseudomonas pseudomallei	23343
Pseudomonas acidovorans	15668-1
Pseudomonas acidovorans	15668-2
Pseudomonas palleronii	17724
Pseudomonas testosteroni	11996
Pseudomonas indoloxidans	9355
Pseudomonas ruhlandii	15749
Pseudomonas stutzeri	17588-2
Pseudomonas mendocina	25411
Pseudomonas alcaligenes	17440
Pseudomonas syringae	19310-1
Pseudomonas syringae	19310-2
Pseudomonas cichorii	10857
Pseudomonas facilis	17695
Alcaligenes paradoxus	17713

Reference Strain	Reference Collection and Strain Number
Pseudomonas delafieldii	17505
Alcaligenes eutrophus	17697
Zoogloea ramigera	19623
Klebsiella pneumoniae	13883
Enterobacter aerogenes	McGill
Serratia marcescens	13880
Pseudomonas alcaligenes	14909
Pseudomonas diminuta	11568
Pseudomonas vesiculare	11426
Pseudomonas maltophilia	13637
Xanthomonas phaseoli	9563
Pseudomonas mallei	23344
Pseudomonas facilis	11228
Aeromonas hydrophila subspecies hydrophila	9071
Aeromonas punctata subspecies caviae	14486
Proteus vulgaris	13315
Escherichia coli	NTCC 9002
Micrococcus roseus	McGill
Alcaligenes fecalis	NTCC 8750
Arthrobacter globiformis	ATCC 8010
Micrococcus luteus	McGill
Pseudomonas caryophylli	ATCC 25418
Cytophaga fermentans	ATCC 19072
Chromobacterium lividum	ATCC 12473
Bacillus subtilis	Harvard
Sphaerotilis natans	ATCC 15291
Azotobacter vinelandii	12837
Bacillus psychrophilis	23304
Sprillillum serpens	12638

Chemical Composition of Media

Collins and Willoughby Medium (1962)

The composition of Collins Medium is as follows:

Bacto peptone	0.5 g
Soluble potato starch	0.5 g
Soluble white casein	0.5 g
Glycerol	1.0 ml
K_2HP0_4	0.2 g
$MgS0_4.7H_20$	0.05 g
$FeCl_3.6H_20$	trace: 4 drops of a 0.01% (W/V) solution
Distilled water	1000 ml
Ph adjusted to	7.2
(Difco) Bacto agar	15.0 g

The agar was dissolved at 100°C for 20 min, then autoclaved at 115°C for 20 min.

Lowe agar, developed by W. E. Lowe, Canada Centre for Inland Waters

The composition of Lowe agar is as follows:

Proteose peptone #3	3.0 g
$MgS0_4.7H_20$	0.05 g
$FeCl_3.H_20$	0.005 g
K_2HP0_4	0.2 g
Ph adjusted to	7.0 with 2N HCl
(Difco) Bacto agar	20 g

The agar was dissolved at 100°C for 20 min.

For Slants. Seven millitres was dispensed into 15 x 125 mm screw cap tubes, autoclaved, then allowed to solidify in the slanted position.

For Plates. The medium was autoclaved at 121°C for 20 min after steaming and poured into plates.

For Semi-solid Tubes. Five grams of Bacto agar, instead of 20 g, was added per litre of distilled water, then steamed at 100°C for 20 min. Five millilitres was dispensed per 16 x 125 mm tube, autoclaved and allowed to solidify.

Basal Medium for Substrate Utilization Tests

The composition of the basal medium (BM) is as follows:

Purified agar	10 g
L-cystine	0.1 g
Distilled water	1000 ml

This was steamed at 100°C for 20 min to dissolve the agar, then sterilized at 121°C for 20 min.

Poly-β-hydroxybutyrate Observation Medium

This medium was made up of two parts:

(A)

$MgS0_4$	2 ml of 10% (W/V) solution
$FeS0_4$	trace, 1 ml of 0.05% (W/V) solution
NH_4S0_4	0.5 g
K_2HP0_4	2.0 g
KH_2P0_4	1.0 g
(Difco) Bacto agar	20 g
Distilled water	900 ml

This solution was steamed for 20 min at 100°C, then autoclaved for 20 min at 121°C.

(B)

Sodium acetate	5 g
Distilled water	100 ml

The sodium acetate solution was sterilized by Millipore filtration and then solutions A and B were mixed. The resulting medium was dispensed into sterile tubes and allowed to harden in the slanted position.

Motility Agar

This medium was of the following composition:

Peptone	10 g
NaCl	5 g
(Difco) Bacto agar	3.5 g
Distilled water	1000 ml

This medium was steamed for 20 min at 100°C. Then 5 mg was dispensed per tube, and autoclaved at 121°C for 20 min.

Tween Hydrolysis Basal Medium.
This medium has the following composition:

(Difco) Proteose peptone #3	3 g
$CaCl_2$	5 g
Distilled water	900 ml
pH adjusted to	7.4
(Difco) Bacto agar	20 g

Steaming for 20 min at 100°C dissolved the medium and autoclaving at 121°C for 20 min sterilized it. Ten grams of each Tween was sterilized in 100 ml of distilled water, then added to the basal medium before pouring.

Bile and Brilliant Green Tolerance

(Difco) Proteose peptone #3	5 g
(BBL) d-mannitol	3 g
Bromocresol purple	0.02 g
Distilled water	1000 ml
(Difco) bile salts	0.5 g
Brilliant green	0.92 g
(Difco) Bacto agar	15 g

This medium was steamed at 100°C to dissolve agar, then autoclaved at 121°C for 20 min.

Chemical Composition of Reagents

Stock Solutions:

(A) Phosphate Buffers

KH_2PO_4	226.8 g
Na_2PO_4	356.6 g
Distilled water	1000 ml

In the original paper of Stanier et al. (1966), the Na and K phosphate concentrations were at 0.033 M but the pH was low. By increasing the sodium phosphate and potassium phosphate concentrations to 0.042 M, the pH was finally raised to pH 7.0. Ten millilitres of this stock solution was dispensed into 15 x 125 mm screw cap tubes, then sterilized by autoclaving at 121°C for 20 min.

(B) Mineral Salts Stock Solution

NH_4Cl	100 g
$MgSO_4.7H_2O$	50 g
$FeCl_3.6H_2O$	0.5 g
$CaCl_2.2H_2O$	0.5 g
Distilled water	1000 ml

The mineral salts stock solution was dispensed into 10-ml volumes and sterilized by autoclaving at 121°C for 20 min.

(C) Vitamin Solution

(a)	Thiamine	0.1 g
	Pantothenate	0.1 g
	Nicotinic acid	0.1 g
	Choline chloride	0.1 g
	Pyridoxamine	0.1 g
	p-Aminobenzoic acid	0.005 g
	Biotin	0.005 g
	Cyanocobalamin	0.1 g
	Methionine	0.8 g
	Distilled water	210 ml
(b)	Riboflavin	1.0 g
	0.02N Acetic acid	20 ml
(c)	Folic acid	0.005 g
	0.02N NaOH	20 ml

The three vitamin solutions, (a), (b), (c) were mixed, Millipore filter sterilized and dispensed into sterile tubes in 3-ml volumes. To prevent oxidation and drying of the vitamins, the tubes were placed in a freezer for storage and thawed just previous to the preparation of the substrate utilization media. Then 3 ml of the vitamin solution was added to one litre of basal medium.

Chemical Reagents:
Cytochrome Oxidase Test

Alpha-naphthol reagent

Alpha-naphthol	1 g
Ethyl alcohol	100 ml

Phenylene Diamine Reagent

N, N-dimethyl-p-phenylene Diamine dihydrochloride	1.0 g
Distilled water	100 ml

H_2S Production Test

Lead Acetate Strips
Chromatography paper strips were soaked in 1% (W/V) solution of lead acetate for 12 to 24 hours. The lead acetate was drained and the strips allowed to dry overnight. They were cut into short pieces and sterilized by autoclaving at 121°C for 20 min before being inserted into the tubes.

Indole Production

Kovac's Reagent

Paradimethylamine benzaldehyde	10 g
Butyl alcohol	150 ml
Concentrated HCl	50 ml

The aldehyde was dissolved in the alcohol, then the acid was added slowly.

Nitrate and Nitrite Reduction Test

(A) Sulfanilic acid solution

Sulfanilic acid	0.8 g
5N Acetic acid	100 ml

(B) Alpha-naphthylamine solution

Alpha-naphthylamine	0.5 g
5N Acetic acid	100 ml

Equal amounts of the above solutions were mixed together, then 0.1 ml was added per tube.

Voges-Proskauer Test

O'Meara Reagent

KOH	40 g
Distilled water	100 ml
Creatine	0.3 g

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