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Microbial characteristics of an industrial mixed liquor after exposure to phenolic compounds

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ABSTRACT

Title of Thesis: Microbial Characteristics of an Industrial Mixed Liquor after Exposure to Phenolic Compounds

Patricia L. Boyle, Master of Science, 1985

Thesis directed by: Dr. Gordon A. Lewandowski
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The microbial characteristics of a mixed population from the Passaic Valley Sewerage Commissioners (PVSC) Wastewater Treatment Plant in Newark were examined. This plant handles about 250 million gallons per day of mixed sewerage, with a large industrial component. The microbial species were characterized using standard plating techniques, morphology, microscopic observation, and biochemical tests. These characteristics were determined for the fresh liquor, and after successive batch exposure to phenol (100 ppm) and 2-chlorophenol (20 ppm). Predator/prey, gram positive to gram negative bacteria ratios, and the fungi and protozoan populations were also determined. These results were compared with those previously obtained using a mixed population from the Livingston (N.J.) Municipal Wastewater Treatment Plant which handles domestic sewerage. There were very few differences between the populations after phenolic exposure, suggesting that the phenomena observed might be generalized, applying to many municipal treatment plants.

Microbial Characteristics of an Industrial Mixed Liquor after Exposure to Phenolic Compounds

by
Patricia L. Boyle

Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the degree of
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Approval Sheet

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This thesis is dedicated to my Parents and Karen with love.

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I. LITERATURE REVIEW

The literature review was conducted using a computer search, beginning with articles published in 1980. Key words included biodegradable materials, phenolic compounds, bacteria, fungi, yeast, and protozoa. 93 articles were identified. The field was narrowed down to 58 articles by chosing only those written in English. Other articles were identified by cross referencing the CAS numbers dealing with the same subject.

Once copies of the articles were obtained, the references in those articles were used as an additional source for the literature search.

The card catalog was also utilized, referencing microbial degradation, biodegradation, activated sludge, and wastewater treatment.

Activated sludge mixed liquor contains a wide variety of microorganisms, of which a large proportion are bacteria (about 10^7/ml) (1,2). Table I-1 shows the total number and viable number of bacteria from different stages of sewage treatment.

Most investigators report that gram negative bacteria predominate over gram positive types (2-6), with the major genera listed in Table I-2. Yeasts are also listed in the table, with numbers reported to be about 10^6 cells/ml for domestic activated sludge (5). Typical protozoan populations are shown in Table I-3, with the majority being ciliates (2,5). Protozoa typically number about $5x10^4$ cells/ml (5). Rotifers and nematodes have also been reported (3), but were not considered to be permanent members of the activated sludge

Table I-1

Numbers of total and viable bacteria in samples from different stages of sewage treatment and in the suspended biomass (2)

Source (and no.) of samples §	Bacterial count* In samples (no./ml)		In biomass† (no./g)		% of bacteria viable
	Total	Viable	Total	Viable	
Settled sewage (22)	$6{\cdot}8 \times 10^{8}$	$1{\cdot}4 \times 10^{7}$	$3{\cdot}2 \times 10^{12}$	$6{\cdot}6 \times 10^{10}$	2·0
Activated sludge mixed liquor (20)	$6{\cdot}6 \times 10^{9}$	$5{\cdot}6 \times 10^{7}$	$1{\cdot}4 \times 10^{12}$	$1{\cdot}2 \times 10^{10}$	0·85
Filter slimes (18)	$6{\cdot}2 \times 10^{10}$	$1{\cdot}5 \times 10^{9}$	$1{\cdot}3 \times 10^{12}$	$3{\cdot}2 \times 10^{10}$	2·5
Secondary effluents (10)	$5{\cdot}2 \times 10^{7}$	$5{\cdot}7 \times 10^{5}$	$4{\cdot}3 \times 10^{12}$	$4{\cdot}7 \times 10^{10}$	1·1
Tertiary effluents (10)	$3{\cdot}4 \times 10^{7}$	$4{\cdot}1 \times 10^{4}$	$3{\cdot}4 \times 10^{12}$	$4{\cdot}1 \times 10^{9}$	0·12

*Counts are geometric means. Total counts using Helber counting chamber, viable counts by plate dilution-frequency method upon CGY agar, incubated at 22° for 6 days (Pike, Carrington & Ashburner, 1972).
†dry wt of suspended solids retained by Whatman GF/C paper.
§from sewage works and laboratory plants treating mainly domestic sewage.

Table I-2

Principal Bacterial and Fungal Genera in Activated Sludge (2,3,5,7)

Gram Positive Bacteria	Gram Negative Bacteria	Fungi
Bacillus	Pseudomonas	Rhodotorula
Micrococcus	Achromobacter	Geotrichum
	Flavobacterium	Candida
	Alcaligenes	Penicillium
	Nitrosomonas	Trichosporon
	Mycobacterium	Pichia
	Brevibacterium	Hansenula
	Nitrobacter	Pullularia
		Phoma
		Cladosporium
		Cephalosporium

Table I-3

Species of Protozoa Commonly Recorded in Activated Sludge (3)

RHIZOPODA Move and ingest food by pseudopodia (Mobile protoplasm)	FLAGELLATA Move by flagella (Whip-like processes)		CILIOPHORA Move by Cilia (Hair-like processes)	
		Free-Swimming	Crawling on sludge floc	Stalked
Ameoba sp.	Bodo caudatus	Paramoecium caudatum	Aspidisca sp.	Acineta sp.
Amoeba actinophora	Cerobodo longicauda	Paramoecium sp.	Euplotes sp.	Podophyra fixa
Arceila vulgaris	Monas sp.	Colidium colpoda	Oxytricha fallax	Vorticella spp.
Actinophyrs sp.	Oikomonas termo	Amphileptus spp.	Stylonychia sp.	Opercularia sp.
Vahlkampfia limax	Euglena sp.	Lionotus fasciola		Epistylis plicatilis
V. guttula	Cercomonas sp.	Chilodon sp.		Carchesium sp.
	Pleuromonas jaculans	Trichoda para		
	Anthophysa vegetans	Loxophyllum sp.		
	Peranema sp.			

population.

Figure I-1 gives an indication of microbial succession during establishment of an activated sludge.

Many of these same bacteria and fungi are capable of degrading phenol and 2-chlorophenol.

Liu and Chapman (7) showed that Alcaligenes, Trichosporon cutaneum, Acinetobacter, and Pseudomonas putida have enzymes (hydroxylases) which degrade various halogenated phenols including phenol and 2-chlorophenol.

Rizzuti and Augugliaro (8) grew Nocardia corallina and Pseudomonas fluorescens, selected from an activated sludge, on agar plates with phenol as the only organic carbon source. They noted that zero order kinetics were followed for biodegradation of phenol. The value of the kinetic rate constant was 0.37 h^{-1} for Nocardia and 0.08 h^{-1} for Pseudomonas.

Anderson et al (1) studied the inhibition and/or toxicity of phenol on unacclimated activated sludge. Toxicity was measured using direct tests such as turbidity and plate counts and indirect tests such as oxygen depletion rates, total organic carbon removal and changes in BOD. Turbidity was measured over time after the addition of phenol at various concentrations. Toxicity was defined as 50% inhibition concentration (IC_{50}). Nontoxic concentrations of phenol were 75-300 mg/l. Some toxicity was noted at 600 mg/l and severe toxicity was seen at 1500 mg/l. Table I-4 shows the pH, temperature, dissolved O_2, Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) in the batch reactor, and Table I-5 shows the number of colony forming units after exposure to phenol.

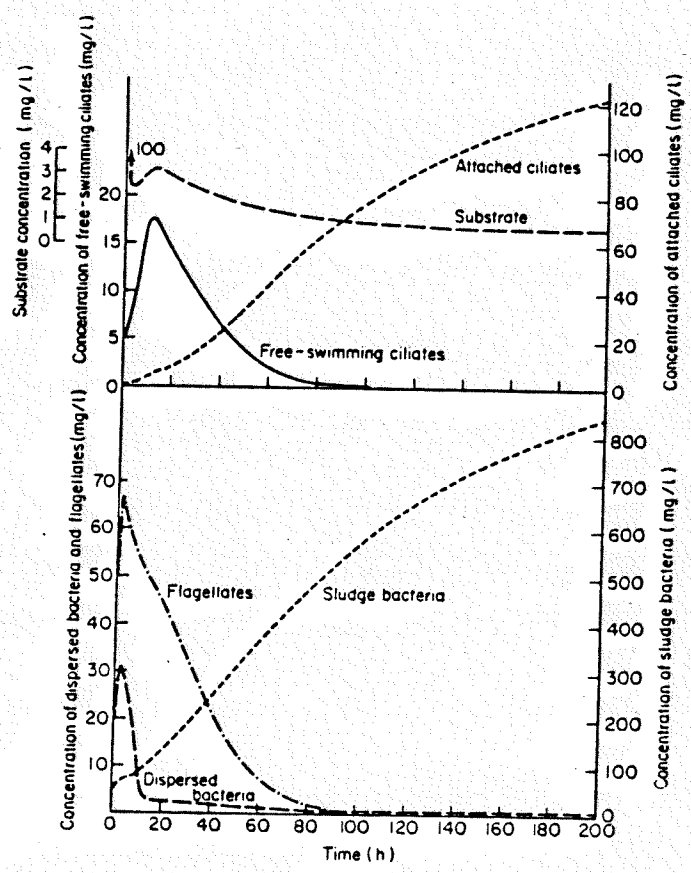


Figure I-1
Computer simulation of successions of micro-organisms during establishment of an activated sludge (2)

Table I-4
Measures of pH, Temperature, Dissolved O_2, Total Suspended Solids, and Volatile Suspended Solids in Batch Reactors (1)

	pH		Temp. (°C)		DO(mg/L)		TSS(mg/L)		VSS(mg/L)	
	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range
Phenol	7.3	6.0-7.7	16.1	15-19	9.9	9.0-10.2	2716	2260-3160	1961	1620-2460
Formaldehyde	7.1	6.0-7.5	19.9	19-22	8.8	8.1-9.2	3099	2720-3480	2008	1960-2300
Complex organic waste + copper sulfate	8.1	7.9-9.2	17.6	16-19	8.9	8.2-9.5	2841	2140-3340	1989	1560-2420

Table I-5

Plate Counts Obtained from Batch Reactors and the Alsop Assay Using Phenol as Test Toxicant (1)

Time (hr)	Toxicant concentration mg/L	Number of colonies per mL	
		Batch reactors	Alsop assay
0	0	46×10^{10}	41.5×10^{10}
	75	42×10^{10}	40.5×10^{10}
	150	46×10^{10}	39.0×10^{10}
	300	43×10^{10}	43.0×10^{10}
	600	55×10^{10}	46.0×10^{10}
	1500	39×10^{10}	41.5×10^{10}
8	0	47×10^{10}	49.5×10^{10}
	75	51×10^{10}	44×10^{10}
	150	37×10^{10}	45.5×10^{10}
	300	50×10^{10}	41×10^{10}
	600	54×10^{10}	45×10^{10}
	1500	33×10^{10}	43×10^{10}
20	0	57×10^{10}	119×10^{10}
	75	62×10^{10}	106×10^{10}
	150	63×10^{10}	104×10^{10}
	300	61×10^{10}	125×10^{10}
	600	68×10^{10}	100×10^{10}
	1500	58×10^{10}	120×10^{10}
28	0	84×10^{10}	108×10^{10}
	75	76×10^{10}	61×10^{10}
	150	61×10^{10}	60×10^{10}
	300	60×10^{10}	84×10^{10}
	600	60×10^{10}	88×10^{10}
	1500	70×10^{10}	$.58 \times 10^{10}$
36	0	80×10^{10}	55.5×10^{10}
	75	68×10^{10}	76.5×10^{10}
	150	52×10^{10}	81×10^{10}
	300	57×10^{10}	74×10^{10}
	600	62×10^{10}	78×10^{10}
	1500	61×10^{10}	80×10^{10}
48	0	92.8×10^{10}	60×10^{10}
	75	80×10^{10}	86×10^{10}
	150	72×10^{10}	98×10^{10}
	300	67×10^{10}	80×10^{10}
	600	64×10^{10}	109×10^{10}
	1500	41×10^{10}	106×10^{10}

Table (continued)

Portier et al (9) did a study where phenol (5 ppm) and 2-chlorophenol (10 ppm) were added to a fresh water microcosm in a continuous flow test. The microcosm consisted of water taken from a location adjacent to a major industrial/petrochemical complex along the river. On phenol addition an increase in bacteria and Actinomyces was observed, with a decrease in fungi, while 2-chlorophenol caused a decrease in all populations. Portier also stated that bacterial and actinomycete populations were responsible for the assimilation of phenol.

Kwasniewska and Kaiser (10) showed that the fermentative yeasts Torulopsis sp. and Rhodotorula sp. were more sensitive to phenol than the oxidative yeasts Pichia and Saccaromyces sp. They also concluded that toxicity to the organism increases with chloro substitutes. Para substituted phenols seemed to be more toxic to yeast, with toxicity increasing with the following substitutions:
$COOH < H < CH_3 < CN < Cl < NO_2$.

Trichosporon cutaneum has been isolated from soil and adapted to use phenol as a major carbon source (11). Other yeasts capable of degrading phenolic compounds are Geotrichum, Saccharomyces, Candida, Rhodotorula, Pullularia, and Oldium. The degradation of phenols by yeast has been reported as limited and slow. Neujahr and Varga (11) reported a reaction scheme for degradation of phenol by Trichosporon cutaneum using phenol hydroxylase and catechol 1,2 oxygenase (see Figure I-2). This ortho pathway is different from comparative studies on the degradation of phenol by Pseudomonas which utilized the meta pathway (12,13).

In determining the structure/toxicity relationship of chloro

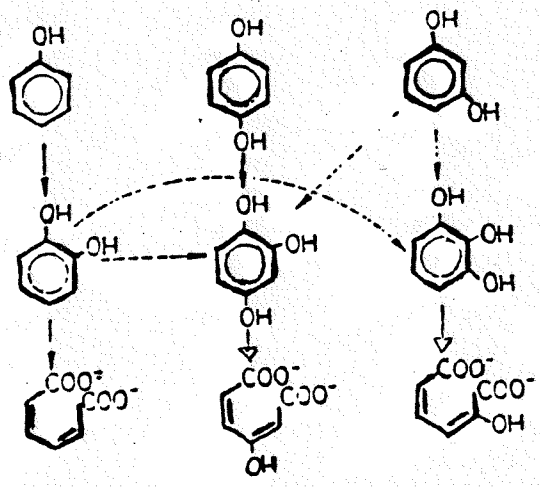


Figure I-2 Proposed pathways of degradation of phenols by T. cutaneum. Reactions demonstrated in: ⟶ Trichosporon; ⇢ Other organisms able to oxidize phenols; ⋯▷ hypothetical reactions (11)

substituted phenols on bacteria, Liu et al (14,28) suggest that the position of the chlorine in phenol affects the chlorophenol toxicity. Para substituted isomers are more toxic than ortho isomers. They also state that ortho isomers of dichlorophenols are less toxic and meta is the most toxic.

Ormerod and Efraimsen (15) presented a toxicity test for evaluation of toxic effects of industrial effluents or chemical substances on the aerobic degradation process. At a concentration of 50 mg/l, phenol was observed as a substrate for the microbial population. At higher concentrations degradation was delayed. The microbial population had to "adapt" to phenol. Figure I-3 shows the dose/response curve for phenol.

Davis et al (16) stated that the microbial population used in the study came from an industrial wastewater treatment unit which was made up of the following bacterial genera: Acinetobacter, Flavobacterium, and Pseudomonas, and the yeast, Rhodotorula. Respiratory inhibition of both the industrial and municipal seed occurred at 50 mg/l of phenol, and degradation was slow. 37 mg/l remained from a 50 mg/l dose after 7 days. One conclusion from a series of toxic exposures to industrial vs. municipal mixed liquors was that limited numbers of microbial genera found in the industrial treatment units performed equally well as large heterogenous generic mixtures in municipal units in responding to individual organic compounds.

Baker et al (17), studied the degradation of chlorophenols in soil, sediment and water. They discovered that 2-chlorophenol, p-chlorophenol, and 2,4-dichlorophenol were degraded by soil and

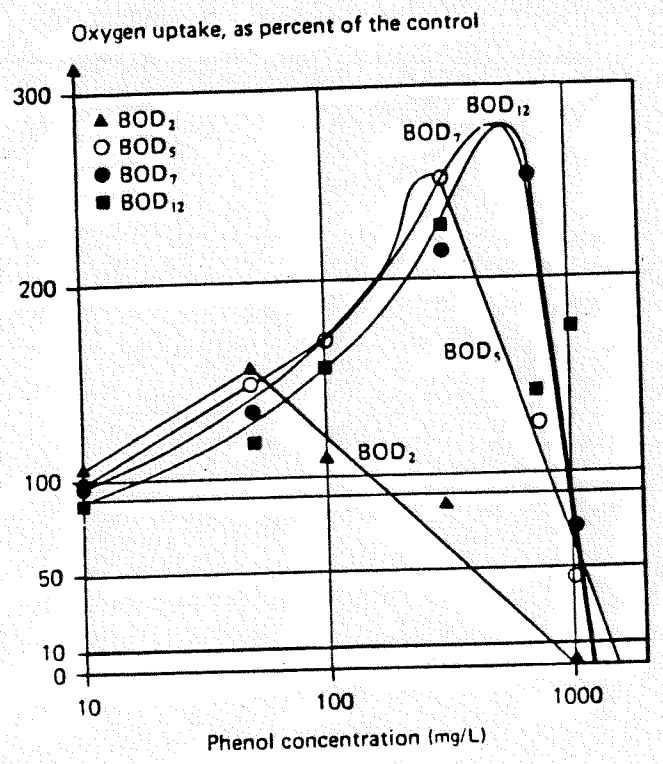


Figure I-3

Dose/response curves for phenol, illustrating increasing adaptation with increasing testing time (15)

sediment microorganisms at 0°C and 4°C. The bacteria Pseudomonas, Xanthomonas, Bacillus, and Flavobacterium have been associated with chlorophenol degradation in soil. Only 2,4-dichlorophenol was degraded by stream water microorganisms at 20°C. The other chlorophenols were not degraded.

Treatment plants considered in the present research were PVSC in Newark, N.J. and Livingston Municipal Treatment Plant in N.J. The Passaic Valley Sewerage Commissioners (PVSC) wastewater treatment plant is one of the country's largest treatment plants for the wastewaters of Northern New Jersey. The influent flow is approximately 260 million gallons per day (MGD), of which the industrial component accounts for approximately 18% by volume and 55% by strength (BOD). The dominant industries are chemical producers, electroplaters, textile dyers, hospitals, electronic products manufacturers, and newsprint recycling mills. The treatment plant responds to an influent wastewater whose pollutant strength is approximately double that of the usual domestic sewage plant with industrial shock loads of 50 to 100% above the average (18). The PVSC wastewater facility secondary treatment utilizes a pure oxygen activated sludge process. The mixed liquor used in the present study comes from the oxygenated activated sludge tank. However, in my work air was used to provide oxygen to the laboratory batch reactors, rather than pure oxygen.

By contrast, the Livingston (N.J.) Municipal Treatment Plant (used for comparison purposes) handles about 3 million gallons per day of almost entirely domestic sewage (99%), and uses conventional aeration (air) in its secondary treatment (i.e. activated sludge) tanks (19).

II. OBJECTIVE

The objective of this research was to characterize the microbial population in the fresh PVSC mixed liquor, and after exposure to phenol and 2-chlorophenol.

PVSC was chosen as the microbial source because of the importance of this facility to wastewater treatment in northern New Jersey. This study can be used as a diagnostic tool to enable extrapolation of results to other systems and to gain more knowledge of a complex catalytic system of which we know very little. The present work is a part of a study to determine treatability limits for industrial organic chemicals for a Publically Owned Treatment Works (POTW).

Phenolic compounds were chosen for several reasons:

(1) high water solubility

(2) low vapor pressure

(3) presence (at much lower concentration) in PVSC influent

(4) in order to compare the results with those using mixed liquor from the Livingston (N.J.) Municipal Treatment Plant (a previous study employed the same compounds).

Concentrations of 100 ppm phenol and 20 ppm 2-chlorophenol used in the present study were based on previous work (20).

III. PROCEDURE

The following experiments were repeated four times to determine reproducibility (designated as runs 1-4).

A. Batch Feeding

Two liters of the Passaic Valley Sewerage Commissioners (PVSC) mixed liquor were put into each of two Lucite batch reactors. There was no mechanical agitation. The aeration rate (500 ml/min), was set so that the mixed liquor did not settle on the bottom of the reactor. The phenol concentration in the first reactor was immediately brought up to 100 ppm, while the second reactor was brought up to 20 ppm 2-chlorophenol. The initial concentration was verified using gas chromatography. When the concentration in each reactor fell below the detection limit (about 1 ppm), they were respiked to their initial concentration (this occurred approximately every day). This process was repeated in each reactor for 10 consecutive days.

Both the initial microbial populations, and those after 10 days exposure, were characterized using various techniques.

After the 10 days, the mixed liquor which had been acclimated to phenol, was then further subjected to additions of 20 ppm 2-chlorophenol for another 10 days. After this second 10 day period, the microbial population was once again characterized. Throughout the experiments, pH and nitrogen (ppm) were determined randomly. The pH range was 6.8 to 7.8, and the nitrogen (as ammonia) varied from 50 ppm to 100 ppm.

The initial exposure of the fresh mixed liquor to 2-chlorophenol will be referred to as "shock-loaded", while exposure after phenol acclimation will be termed "acclimated".

B. Characterization Techniques

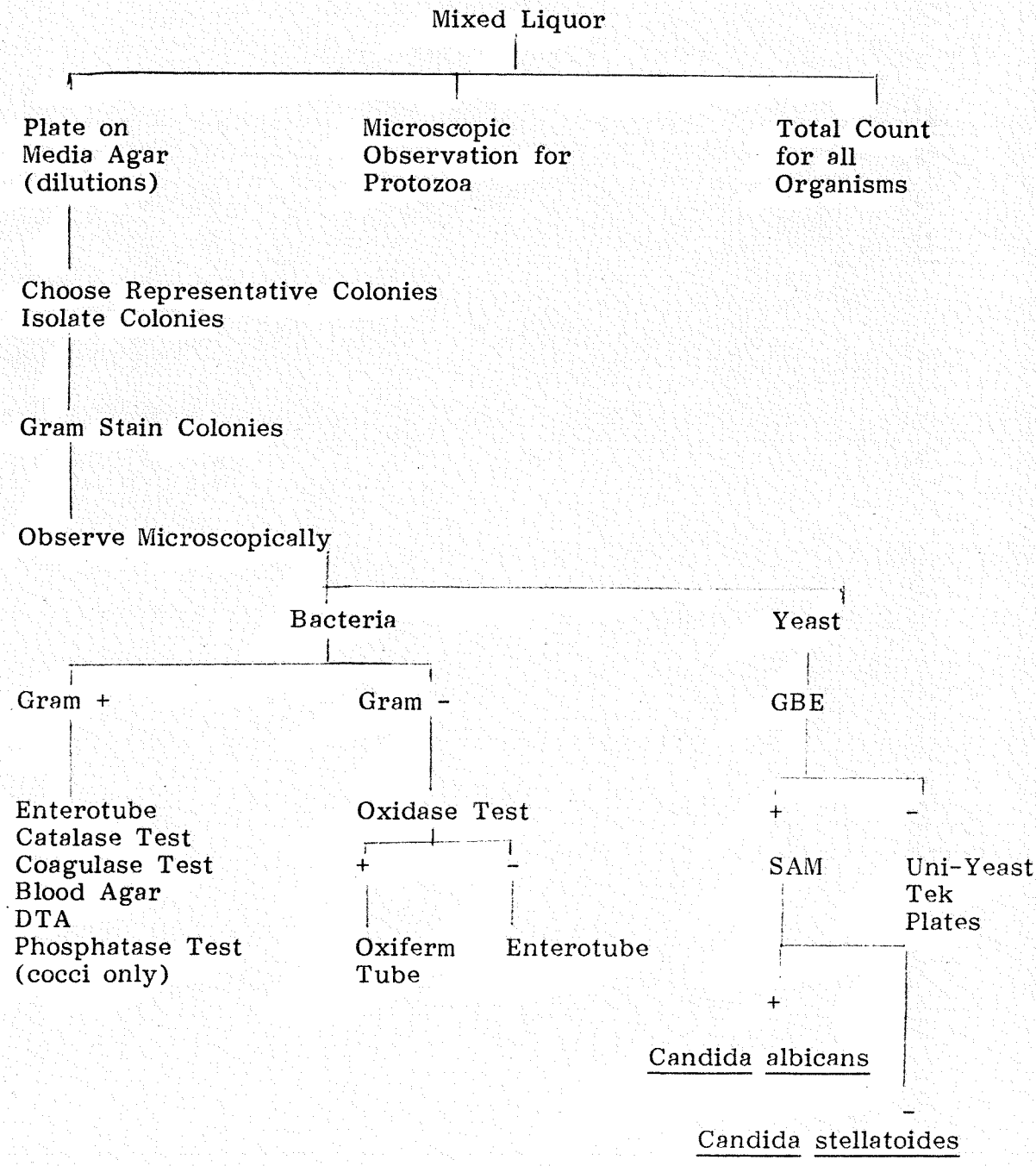
(details may be found in Appendix A and B)

Table III-1 shows the procedure for identification of microbes in the PVSC mixed liquor. After the 10 day period of addition of phenol or 2-chlorophenol, a 10ml sample was taken from each reactor and placed in separate 25ml glass vials with 5mm glass beads. The vials were shaken 25 times to separate bacterial clumps. Using 50 microliters of the shaken mixed liquor, taken from the vial, an organism count was obtained using a Petroff-Hauser counting chamber (See Appendix B). The rest of the sample was then reshaken and a series of dilutions were made. A 10^{-1} dilution was made using 0.5 ml of the mixed liquor pipetted into 4.5 ml of 0.1% Tween 80. Tween 80 was added for the dispersion of fungal spores. The rest of the dilutions (10^{-2} to 10^{-12}) were made with sterile distilled water. Selected dilutions (10^{-10}, 10^{-11}, 10^{-12}) were used for bacterial characterization, and the 10^{-1}, 10^{-3}, 10^{-5} dilutions were used for yeast and mold characterization.

Differential media, selective media, and general growth media were prepared for bacterial and fungal cultures. Differential media supports the growth of various species, while providing an environment that makes it easier to distinguish between different species. An example of differential media is Blood Agar and the

TABLE III-1

PROCEDURE FOR IDENTIFICATION OF MICROBES IN PVSC MIXED LIQUOR



ability to differentiate Staphylococcus sp. and Streptococcus sp. If the bacteria shows hemolysis on the blood agar plate, it is identified as Streptococcus pneumoniae. If hemolysis occurs and the colonies are large and opaque, Staphylococcus aureus has been isolated. Selective media interferes with or prevents growth of certain organisms while permitting growth of others. Certain agents are added to the media to prevent growth (i.e. antibiotics or HCl to inhibit bacteria while permitting the growth of fungi). Plate Count (Difco) was the general growth media used for bacterial cultures. Selective media used were cellulose, floc, Thiobacillus and N_2. Blood agar and phosphatase media were the differential media used for bacterial cultures. (See Appendix B) For yeasts and molds, the selective media used were Czapek (Difco) with rose bengal, Sabouraud (Difco), YM agar, YNB with sucrose, and OXA agar. These plates, excluding the differential media were inoculated with 0.1 ml of the selected dilutions and spread over the surface of the plate with a sterile glass spreader. The plates were incubated for 72 hours at room temperature (25°C). Blood agar and phosphatase plates were incubated for 24 hours at 37°C. (See Appendix B)

1. Bacteria

After incubation the bacterial plates were then counted for the number of different bacteria. Size, shape, and color of each colony were recorded to be used later for identification purposes. Then a representative of each different bacterial colony was isolated using a streak plate method (See Appendix B). Once the bacterial species

was isolated, further tests were performed to identify the particular bacteria.

A Gram stained slide of each representative bacteria determined which were Gram positive (purple) or Gram negative (red). Also noted were the shape of the organism (rod or cocci), as well as the relative size of the organism. Gram positive bacteria were then subjected to the catalase test (indicating the presence of catalase enzyme) and coagulase test (presence of coagulase enzyme), followed by inoculation on dextrose tryptone agar (DTA), blood agar, and Enterotubes (Hoffmann-LaRoche). The DTA, blood agar, and Enterotubes were then incubated for 24 hours at 37°C. The results were recorded, and Bergey's Manual (21) was used to identify the bacterial species.

Gram negative bacteria were subjected to an oxidase test (See Appendix B). If the results were positive (presence of dark purple color) an Oxiferm tube (Hoffmann-LaRoche) was inoculated; otherwise, an Enterotube was inoculated. After a 48 hour incubation at 37°C, the results were recorded and the bacterial species was identified using code books supplied by Hoffmann-LaRoche (22,23).

If the results of the biochemical tests (Enterotube or Oxiferm tubes) suggested more than one bacterial species, confirmatory test were performed to narrow the selection to one bacterial species. These tests included sensitivity to various antibacterial agents, sensitivity to various temperatures, color of bacterial colonies, location of flagella, etc.

2. Fungi

The molds were identified by morphology and color. The particular mold in question was observed under the microscope, and size and shape of the spores and morphology of hyphae were noted. Together with the color of the mold top and bottom and its growth on the plate, the genus of the mold was identified using a reference book (24).

The yeasts were also observed under the microscope to note their reproductive stages. The yeasts were also isolated (like the bacteria) using the streak plate technique. The isolated yeast then underwent further biochemical tests. The 18-24 hour old yeast was inoculated into a GBE tube (Flow Lab). GBE is a growth medium that brings about germination of particular yeast spores. After the GBE/yeast mixture was incubated for 6 hours at 38°C, a drop of the mixture was placed under the microscope. If presence of germ tubes was observed the yeast identification was narrowed down to two possible candidates, Candida albicans or Candida stellatoides.

The germ tube positive yeast was then inoculated onto a SAM Slant (Flow Labs). The SAM tube contains a sugar which can be utilized by C. albicans but not C. stellatoides. Metabolism of the sugar by C. albicans will change the color of the Slant from purple to yellow after 24 hour incubation at 28-30°C.

If the yeast were observed to be germ tube negative then a suspension of the yeast cells was made using sterile distilled water. This suspension was then inoculated onto a UNI-YEAST TEK plate and then kept at room temperature for 6 days. UNI-YEAST TEK plates

contain 11 wells. Each well contains a medium with a pH indicator. If the yeast utilizes the substrate in the medium, metabolic products are produced, the pH changes, and a color change is observed. The color changes are coded and the yeast identified according to a code book supplied by Flow Laboratories (25).

3. Protozoa

Using the Petroff-Hauser Counting Chamber, the number of protozoa per cubic centimeter was obtained. To prevent the protozoa from moving under the microscope formalin was added to the mixed liquor on the slide. Identification was accomplished by noting the size, morphology, and type of locomotion of the protozoa (26).

IV. RESULTS

A. Microbial Counts

The number (per cm^3) of bacteria, yeast and protozoa decreased in the mixed liquor with exposure to phenol and 2-chlorophenol. (See Tables IV-1, IV-2) The bacteria and yeast in the mixed liquor showed a decrease of one order of magnitude after initial exposure to phenol or 2-chlorophenol. After phenol acclimation, yeast numbers anomalously increased on further exposure to 2-chlorophenol. Surprisingly, the protozoa count remained constant during phenolic exposure. These anomalies will be discussed in Section V.

B. Characterization Test Results

Appendix C shows the bacterial and yeast characterization test results for all four runs. Seasonal variations were taken into account during this study. The mixed liquor, for the four replicate experiments, was taken successively from PVSC during July, August, November (1984), and end of January (1985). Nevertheless, results were very consistent from run to run. Organism counts never varied by more than a factor of 1.4, nor did gram negative/gram positive ratios vary by more than a factor of 1.5. Predominant genera appeared consistently, although species sometimes varied. If a particular organism was found in only one run it was noted as such. Table IV-3 show the bacterial species found in PVSC mixed liquor before and after exposure to phenol and 2-chlorophenol. While using the Oxiferm Code Book,"name equivalent" for oxidative-fermentative gram-negative rods were used because standardization of nomenclature

TABLE IV-1

PREDOMINANT MICROBIAL GENERA IN PVSC MIXED LIQUOR ON SUCCESSIVE PHENOLIC EXPOSURE

Fresh Mixed Liquor	Phenol Acclimated*	Acclimated 2-Chlorophenol**
10^9 bacteria/cm³	10^8 bacteria/cm³	10^8 bacteria/cm³
10^6 yeast/cm³	10^5 yeast/cm³	10^6 yeast/cm³
10^5 protozoa/cm³	10^5 protozoa/cm³	10^5 protozoa/cm³
Gram positive / Gram negative = 1	0.3	0.3
Gram positive cocci (Micrococcus)	X	X
Gram positive rods (Bacillus)	X	X
Pseudomonas	X	X
Xanthomonas	X	X
Enterobacter	X	
Alcaligenes	+	
Acinetobacter	X	X
Pasturella		+
Providencia	X	
Staphylococcus+		
Aspergillus	X	X
Penicillium	X	X
Trichophyton		
Geotrichum		
Candida	X	X
Debaromyces	X	X
Cryptococcus	X	X
Trichosporon	X	X
Saccharomyces		
Rhodotorula		
Streptomyces	X	X
Hansenula+		
Epistylis	X	X
Opercularia	X	X
Carchesium		
Stylonychia		
Paramecium		
Peranema	X	X
Colpidium	X	X
Podophyra		

* 100 ppm for 10 days
** followed by 20 ppm for 10 days
\+ not dominant genera

TABLE IV-2

PREDOMINANT MICROBIAL GENERA IN PVSC MIXED LIQUOR-
COMPARISON OF RESULTS FOR 2-CHLOROPHENOL

Fresh Liquor	Shock load 2-Chlorophenol*	Acclimated 2-Chlorophenol
10^9 bacteria/cm^3	10^8 bacteria/cm^3	10^8 bacteria/cm^3
10^6 yeast/cm^3	10^5 yeast/cm^3	10^6 yeast/cm^3
10^5 protozoa/cm^3	10^5 protozoa/cm^3	10^5 protozoa/cm^3
Gram positive / Gram negative = 1	0.5	0.3
Gram positive cocci(*Micrococcus*)	X	X
Gram positive rods(*Bacillus*)	X	X
Pseudomonas	X	X
Xanthomonas	X	X
Enterobacter		
Alcaligenes		
Acinetobacter	X	X
Pasturella		+
Providencia		
Staphylococcus+		
Aspergillus	X	X
Penicillium	X	X
Trichophyton		
Geotrichum		
Candida	X	X
Debaromyces		X
Cryptococcus	X	X
Trichosporon	X	X
Saccharomyces		
Rhodotorula		
Streptomyces		X
Hansenula+		
Epistylis	X	X
Opercularia	X	X
Carchesium		
Stylonychia		
Paramecium		
Peranema	X	X
Colpidium	X	X
Podophyra		

* 20 ppm for 10 days
\+ not dominant genera

Table IV-3
Bacterial Species found in PVSC Mixed Liquor

Fresh Mixed Liquor	Phenol (100 ppm for 10 days)	Acclimated 2-Chlorophenol (20 ppm for 10 days)	Shock-loaded 2-Chlorophenol (20 ppm for 10 days)
Micrococcus	Micrococcus	Micrococcus	Micrococcus
Bacillus	Bacillus	Bacillus	Bacillus
Pseudomonas cepacia	Pseudomonas cepacia	Pseudomonas cepacia	Pseudomonas cepacia
P. vesicularis	P. aeruginosa	P. vesicularis	2k-1 Pseudomonas like+
P. aeruginosa	P. fluorescens	P. maltophilia *	Acinetobacter lwoffi
P. fluorescens	2k-1 Pseudomonas like+	2k-1 Pseudomonas like+	Acinetobacter anitratus
P. putida	5E-1 Pseudomonas like	5E-1 Pseudomonas like	
2k-1 Pseudomonas like+	E. agglomerans	Acinetobacter sp	
5E-1 Pseudomonas like	Alcaligenes faecalis	Pasturella ureae	
Enterobacter agglomerans	Acinetobacter lwoffi	Achromobacter *	
Alcaligenes faecalis	Acinetobacter anitratus		
4E Alcaligenes like	Providencia stuarti		
Acinetobacter lwoffi			
Acinetobacter anitratus			
Pasturella ureae			
Providencia stuarti			
Staphylococcus aureus			
Citrobacter freundii *			
Moraxella sp. ?*			

\+ also known as Xanthomonas
* only found in one run of the experiment
? not positively identified

is not always seen. While one genera is identified by the (Code Book)it could also be named by other genera that have the same biochemical reactions (22). An example of this is Group 2K pseudomonas-like also being identified as Xanthomonas; and another name for Acinetobacter anitratus is Achromobacter anitratum. Therefore, one must be aware of the various differences in nomenclature while identifying bacteria.

1. Gram Positive/Gram Negative Ratios

Tables IV-1, IV-2 show the gram positive/gram negative ratio for the fresh mixed liquor, phenol acclimated, 2-chlorophenol acclimated, and 2-chlorophenol shock-loaded populations.

The fresh mixed liquor had a gram positive/gram negative ratio of approximately 1. After exposure to phenolics the gram negative bacteria dominated by a factor of 3.

2. Gram Positive Bacteria

Appendix C shows the biochemical test results for gram positive bacteria. Morphologically the colonies were flat, dull, cream color with scalloped margins and a concentric configuration. The rod-shaped bacteria identified, using Bergey's Manual, (21) were Bacillus.

Tiny cocci were also observed. Morphologically the colonies were white, smooth, opaque, and convex with a smooth margin and round configuration. These organisms may be Micrococcus.

During exposure to phenol and 2-chlorophenol the number of gram positive bacteria decreased. Gram negative bacteria such as Pseudomonas, Acinetobacter, and Xanthomonas, persist throughout the exposures.

3. Fungi

Table IV-4 show the molds and yeasts identified in the fresh mixed liquor, and after exposure to the phenolics.

Phenol acclimated mixed liquor had a decrease in the number of yeast present but the diversity was still similar with the exception of Saccaromyces, Rhodotorula and Hansenula. Surprisingly, 2-chlorophenol acclimated mixed liquor showed an increase in the number of yeast cells over the phenol acclimated mixed liquor (but with the same diversity). The apparent increase in the number of yeast cells after 2-chlorophenol acclimated exposure will be discussed later.

Shock-loading the fresh mixed liquor with 2-chlorophenol reduced the number of yeast cells and eliminate one of the genera.

4. Protozoa

PVSC had a variety of protozoa in the mixed liquor. In the fresh mixed liquor, stalked, flagellated, and ciliated protozoa were seen.

After exposure to the phenolics, the total number of protozoa stayed the same, but the flagellated and non-stalked ciliates decreased. Stalked ciliates dominated after exposure to phenolics, but the stalked ciliates broke apart and were erroneously counted as separate organisms which could account for the protozoa population remaining stable when ,in fact, it had decreased. Tables IV-1 and

TABLE IV-4

Fungal Species Found in PVSC Mixed Liquor

Fresh Mixed Liquor	Phenol (100 ppm for 10 days)	Acclimated 2-Chlorophenol (20 ppm for 10 days)	Shock-Loaded 2-Chlorophenol (20 ppm for 10 days)
Aspergillus sp	Aspergillus sp	Aspergillus sp	Aspergillus sp
Penicillium sp	Penicillium sp	Penicillium sp	Penicillium sp
Trichophyton sp	Candida albicans	Candida sp	Candida krusei
Geotrichum sp	Debaromyces hansenii	Debaromyces hansenii	Cryptococcus sp
Candida sp	Cryptococcus sp	Cryptococcus sp	Trichosporon pullulans
Candida albicans	Trichosporon beigleii	Trichosporon beigleii	
Candida famata	Streptomyces	Streptomyces	
Debaromyces hansenii			
Cryptococcus sp			
Trichosporon sp			
Saccharomyces cerevisiae			
Rhodotorula rubra			
Streptomyces			
Hansenula anomala			

IV-2 show the protozoa observed before and after exposure to phenol and 2-chlorophenol.

V. DISCUSSION

In the PVSC mixed liquor, bacterial and yeast counts decreased after exposure to phenolics by an order of magnitude. This is probably a result of the high initial microbial concentration in the fresh mixed liquor, which existed in a rich nutrient environment (sewage). By contrast, the phenolics represented a more toxic food source, at a lower concentration. In addition, during 10 days exposure in a batch reactor toxic metabolic products could build up, contributing to a decrease in microbial population.

Surprisingly, the total protozoa population seems to remain constant. Upon exposure to phenol, the free swimming protozoa population decreased. Figure V-I shows succession of dominant protozoa in relation to organic waste. These results are consistent with the findings of Pike and Curds(2) and Hawkes (3). Stalked ciliates have a number of branches on each stalk, although they were counted as a single organism. When the environment was stressed (addition of phenolics) the stalks were observed to break apart. Under the microscope, the broken stalks were counted as individual ciliates. This would account for the protozoa population apparently remaining constant, even though their food supply (bacteria) decreased.

The gram negative to positive ratios became greater than one after exposure to the phenolics. These results are consistent with the literature described previously. Although two gram positive species (Bacillus and Micrococcus) have been shown to degrade phenols, there are many more gram negative species that are known to be phenol degraders. (1-8,12-17).

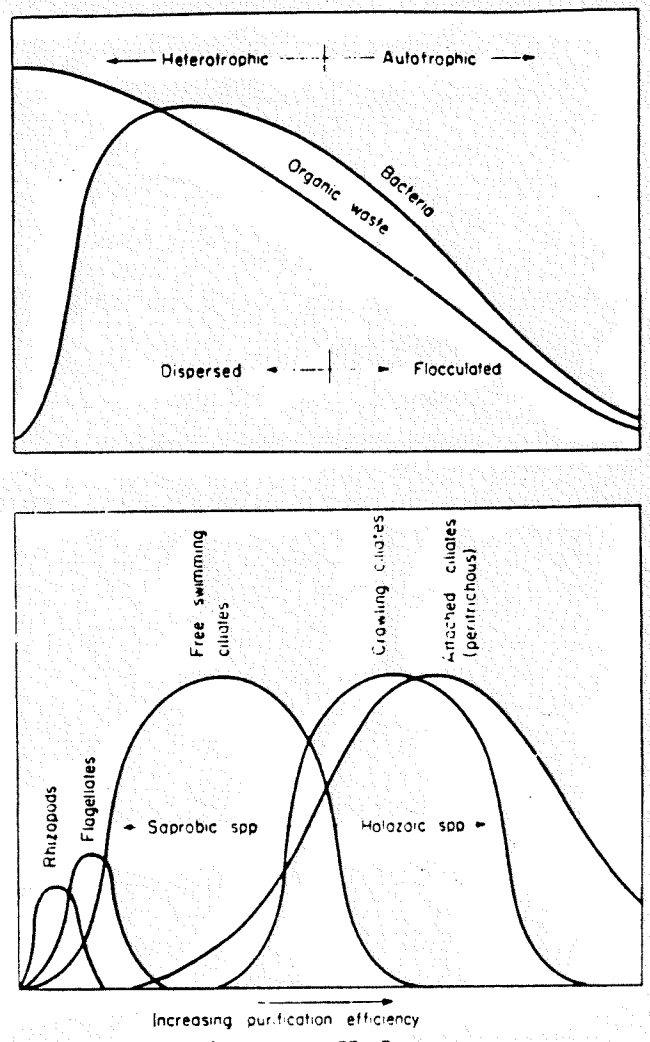


Figure V-1

Hypothetical curves showing successions of dominant protozoa in relation to the degree of purification of organic waste and bacterial population (3)

The results of shock-loading the PVSC mixed liquor with 2-chlorophenol were compared to those with prior acclimation to phenol. The only difference observed in bacteria populations was the presence of Pasturella ureae found in the acclimated 2-chlorophenol mixed liquor but not in the shock-loaded mixed liquor (although the acclimated 2-Chlorophenol had a larger diversity of pseudomonads). Since shock loading creates a more stressful environment for the microorganisms this variation was expected. The only other difference between the two exposures were the number of yeast present. For acclimated 2-chlorophenol it was an order of magnitude higher. Conditions favorable to yeast (over bacteria) include low pH and nitrogen levels. However, the pH was 6.8 to 7.8 (which is consistent with other work in the laboratory), and nitrogen varied from 50 ppm to 100 ppm (which is more than sufficient). Protozoa feed on bacteria, rather than yeast (27), but the yeast population decreased anyway on phenol exposure; so this would not account for the apparent increase in yeast population. However, this rise was observed consistently for four runs. The answer may be that the yeast population after 20 days is the first to recover from phenolic exposure.

Tables V-1 and V-2 compare the results of this study (using PVSC mixed liquor) to a study done previously by Laurie Gneiding on Livingston mixed liquor (20). PVSC is a 260 MGD plant with a 55% industrial component, and utilizes pure-oxygen activated sludge tanks. Livingston is a 4 MGD plant with 99% domestic waste and utilizes conventional aeration.

TABLE V-1

PREDOMINANT MICROBIAL GENERA IN LIVINGSTON MIXED LIQUOR

Fresh Liquor	Phenol Acclimated	Acclimated 2-Chlorophenol
10^{11} bacteria/cm³	10^{8} bacteria/cm³	10^{8} bacteria/cm³
10^{6} yeast/cm³	10^{6} yeast/cm³	10^{6} yeast/cm³
10^{5} protozoa/cm³	10^{5} protozoa/cm³	10^{5} protozoa/cm³
$\frac{\text{Gram positive}}{\text{Gram negative}} = 1$	25	25
Gram positive cocci(*Micrococcus*)	X	X
Gram positive rods (*Bacillus*)	X	X
Pseudomonas	X	X
Xanthomonas	X	X
Enterobacter	X	
Alcaligenes	X	X
Achromobacter	X	X
Acinetobacter	X	X
Serratia	X	
Aeromonas		
Escherichia coli		
Pasturella		
Penicillium	X	X
Trichophyton		
Geotrichum		
Candida	X	X
Debaromyces	X	
Cryptococcus	X	X
Trichosporon	X	
Saccharomyces		
Rhodotorula		
Streptomyces	X	
Epistylis	X	X
Colacium	X	X
Lionotus	X	
Mayorella	X	X
Valkampfia	X	
Aspidisca	X	X
Oxytricha		
Polychaos		
Peranema		
Paramecium	X	
(20/cm³) Rhabiditoideae (nematode)	X	X
Philodina (rotifer)	X	X
Tyrelia (water mite)	X	X

TABLE V-2

PREDOMINANT MICROBIAL GENERA IN PVSC MIXED LIQUOR ON SUCCESSIVE PHENOLIC EXPOSURE

Fresh Mixed Liquor	Phenol Acclimated*	Acclimated 2-Chlorophenol**
10^9 bacteria/cm³	10^8 bacteria/cm³	10^8 bacteria/cm³
10^6 yeast/cm³	10^5 yeast/cm³	10^6 yeast/cm³
10^5 protozoa/cm³	10^5 protozoa/cm³	10^5 protozoa/cm³
Gram positive / Gram negative = 1	0.3	0.3
Gram positive cocci (Micrococcus)	X	X
Gram positive rods (Bacillus)	X	X
Pseudomonas	X	X
Xanthomonas	X	X
Enterobacter	X	
Alcaligenes	+	
Acinetobacter	X	X
Pasturella		+
Providencia	X	
Staphylococcus+		
Aspergillus	X	X
Penicillium	X	X
Trichophyton		
Geotrichum		
Candida	X	X
Debaromyces	X	X
Cryptococcus	X	X
Trichosporon	X	X
Saccharomyces		
Rhodotorula		
Streptomyces	X	X
Hansenula+		
Epistylis	X	X
Opercularia	X	X
Carchesium		
Stylonychia		
Paramecium		
Peranema	X	X
Colpidium	X	X
Podophyra		

* 100 ppm for 10 days
** followed by 20 ppm for 10 days
\+ not dominant genera

(1) Bacterial counts in both mixed liquors showed similar trends when exposed to phenolics. Livingston had an initially higher bacterial count than PVSC (by a factor of 100). A repeat of bacterial counting confirmed these results (37). However, mixed liquor suspended solids (MLSS) in the PVSC plant was about 5000 mg/l, while it was only 2500 mg/l at Livingston. Thus, the viable population was totally unrepresented by MLSS. One reason for the difference in numbers could be errors in counting. Under the microscope debris and pieces of organic matter can be mistaken for bacteria. Also bacteria tend to form clumps and the mixture should be shaken vigorously 25 times to break up these clumps.If this was not done properly, it could account for lower numbers.

(2) In the Livingston activated sludge, the gram positive were reported to dominate over gram negative bacteria 25 to 1 after exposure to phenolics(20). However, gram stained microorganisms are difficult to distinguish properly under the microscope. More recent work in the same laboratory (37) has indicated that the Livingston sludge may indeed exhibit a decreasing gram positive / gram negative ratio, which would be consistent with the present study (and with the literature).

(3) The variety of bacteria found in PVSC mixed liquor was very similar to those found in Livingston mixed liquor. The organisms not found in PVSC but found in Livingston were Aeromonas hydrophila, Serratia liquefaciens, Achromobacter sp., and, Escherichia coli, an organism found in fecal waste. PVSC does have

a domestic waste source, so E. Coli should be present. But, since PVSC receives phenol at ppb levels, it may be that E. Coli does not survive the exposure prior to the activated sludge tanks. It similarly did not survive on phenol exposure in Laurie's work (20). On the other hand, the only organism identified in PVSC but not in Livingston was Providencia stuartii. These differences could be attributed to differences between samples from the same treatment plant. In Laurie's work, the study was done only once but the PVSC study was repeated four times with consistent results.

(4) After exposure to phenol (100 ppm) for ten days, the bacterial populations in PVSC and Livingston differed only slightly. The organisms not present in PVSC were Achromobacter and Serratia (which were not found in the fresh PVSC mixed liquor). After further exposure to 2-chlorophenol, the only organism not found to persist in PVSC but found in Livingston was Alcaligenes.

(5) In comparing the fungi present in PVSC vs. Livingston mixed liquor, the primary difference was the greater persistence of PVSC yeast genera (Debaromyces and Trichosporon) on 2-Chlorophenol exposure. In addition, Aspergillus and Hansenula were seen in the fresh mixed liquor of PVSC but not in Livingston. However, Aspergillus was noted as a contaminant in L. Gneiding's work (20).

(6) The protozoa populations differed quite a bit. Livingston seemed to have a more diverse population of protozoa, with a variety

of ameboids, flagellates, and ciliates. After exposure to phenolics in the Livingston mixed liquor, ameboids (Mayorella) began to dominate over stalked ciliates with an occasional flagellate (Aspidisca) seen. In PVSC, the stalked ciliates predominated after exposure to the phenolics. Many of the protozoa seen in Livingston are morphologically similar to those seen in PVSC mixed liquor. Therefore, differences in the observations by two investigators could account for the differences in the protozoan population.

Overall, the populations in the PVSC and Livingston mixed liquors were surprisingly similar. Livingston did seem to have a slightly larger variety of microorganisms in the fresh mixed liquor but the populations after phenolic exposure were very similar. Since there was very little industrial component (less than 1%) in Livingston, the fresh mixed liquor bacterial population may not have been as constrained as much as in PVSC.

VI. CONCLUSION

The microbial characteristics of a mixed population from the PVSC Wastewater Treatment Plant in Newark were examined before and after exposure to phenolic compounds. As seen throughout the experiment, the PVSC mixed liquor showed a wide diversity of microorganisms (including protozoa) even after exposure to phenolics.

Gram negative bacteria dominated over gram positive bacteria after phenolic exposure. The dominant gram negative genera were Pseudomonas, Xanthomonas and Acinetobacter. Bacillus and Micrococcus were the dominant gram positive genera.

Dominant molds in the fresh mixed liquor, and persisting through phenolic exposure, included Asperigillus and Pencillium while the dominant yeasts were Candida , Cryptococcus , and Trichosporon .

The protozoan population changed after exposure to the phenolics. Free swimming ciliates and flagellates decreased. The stalked ciliates, Epistylis and Opercularia dominated in the mixed liquor after exposure to phenol and 2-chlorophenol.

In comparing the PVSC mixed liquor to Livingston mixed liquor, the variety of organisms were very similar even after phenolic exposure. This in spite of the large differences between the feed and operation of the two plants. The major difference observed was the reported dominance of gram positive bacteria in the Livingston mixed liquor (as opposed to dominant gram negative bacteria in PVSC).A recent repeat of the Livingston characterization tests(37) showed gram negative bacteria dominating, as in PVSC. The gram

negative dominance is in agreement with the literature review. The other difference was protozoan populations. A greater variety was found in Livingston mixed liquor, with the ameboid type dominating after phenolic exposure, whereas PVSC showed continuing dominance of stalked ciliates.

APPENDIX A

MEDIA AND STAINS (29,30,31,32)

Gram Stain
Crystal Violet
Gram Iodine
Safranin
Decolorizer

Procedure: Place a small amount of an 18-24 hour old isolated bacterium on a slide with a drop of sterile distilled water. Let the smear air dry. Heat fix the smear. Flood the slide with crystal violet and let remain one minute. Wash off excess with distilled water. Flood slide with gram iodine, let it remain for one minute. Wash off with distilled water. Flood with decolorizer until the solvent runs colorless from the slide. Wash with distilled water. Flood with Safranin and let it remain one minute. Wash with distilled water. Let dry. The slide is ready for observation under the microscope.

Leifson Flagella Stain

Solution A: Dissolve 0.9 grams of pararosaniline acetate and 0.3 grams of pararosaniline hydrochloride into 100ml of 95% ethyl alcohol. Let it stand overnight at room temperature to completely dissolve.

Solution B: Dissolve 3.0 grams of tannic acid in 100ml distilled water.

Solution C: Dissolve 1.5 grams of NaCl in 100ml of distilled water.

Procedure: Mix equal volumes of solutions A, B, and C and let stand for 2 hours. Store in a stoppered bottle in refridgerator (2 months). Disregard precipitate that forms in in the bottle. Do not filter. It can be stored indefinitely if frozen.

Blood Agar

Blood Agar Base (Difco)	40 grams
Distilled Water	1 liter

After cooling agar to 45°C add 5% citrated sheep blood.

Brain Heart Infusion Broth (Used to grow organisms for Flagella Stain)

Brain heart infusion powder	37 grams
Distilled Water	1 liter

Pour into sterile test tubes and plug the tops. Sterilize in autoclave at 121°C for 15 minutes.

Nutrient Agar (Difco)

Nutrient Agar	23 grams
Distilled Water	1 liter

Plate Count Agar

Plate Count Agar	23.5 grams
Distilled Water	1 liter

Cellulose Decomposing Bacteria Medium

$(NH_4)_2SO_4$	0.10 grams
K_2HPO_4	0.10 grams
$MgSO_4*7H_2O$	0.05 grams
$CaCO_3$	0.20 grams
NaCl	trace
Agar	2.00 grams
Distilled Water	100 ml

Autoclave when cool, pour into plates and add a strip of filter paper.

Centrimide Agar

Gelysate pancreatic digest of gelatin	2.00 grams
$MgCl_2$	0.14 grams
K_2SO_4	1.00 grams
Agar	1.40 grams
Cetrimide	0.03 grams
Distilled Water	100 ml

Czapek (or Cornmeal) Agar with Rose Bengal

Czapek powder	4.9 grams
Rose Bengal	33.0 grams
Distilled Water	100 ml

Dextrose Tryptone Agar

Tryptone	1.00 grams
Dextrose	0.50 grams
Agar	1.50 grams
Bromcresol purple	0.004 grams
Distilled Water	100 ml

Floc-Forming Bacteria Medium

Proteose - Peptone	0.20 grams
Yeast Extract	0.10 grams
Agar	2.00 grams
Distilled Water	100 ml

Motility Agar

Beef Extract	0.30 grams
Peptone	1.00 grams
NaCl	0.50 grams
Agar	0.40 grams
Distilled Water	100 ml

Nitrifying Bacteria Medium

$(NH_4)_2SO_4$	0.005 microliters
NaCl	0.005 microliters
KH_2PO_4	0.001 microliters
$MgSO_4*7H_2O$	0.001 microliters
$CaCO_3$	1.080 grams
Agar	2.080 grams
Distilled Water	100 ml

Autoclave. After plates are inoculated, incubate in the dark at 25°C for 1-2 weeks.

OXA Medium

Peptone	10.0 grams
Glucose	10.0 grams
Oxgall (bovine bile)	15.0 grams
Agar	20.0 grams
Distilled Water	1 liter

Add 50mg of streptomycin and 10mg of penicillin G . After autoclaving filter, sterilize the antibiotics.

Sabouraud Medium

Peptone	1.00 grams
Glucose	4.00 grams
Agar	1.50 grams
Distilled Water	1 liter

Autoclave. After plates are inoculated, incubate the plates in the dark at 25°C for 1-2 weeks.

YM Agar

Yeast Extract	0.30 grams
Malt Extract	0.30 grams
Peptone	0.50 grams
Glucose	1.00 grams
Agar	2.00 grams
Distilled Water	100 ml

Autoclave. Cool to 50°C, then add 0.70ml of 1N HCl.

YNB Agar with Sucrose

Yeast Nitrogen Base Powder	6.70 grams
Sucrose	5.00 grams
Agar	1.50 grams
Distilled Water	1 liter

Appendix B

Procedures used in characterization of organisms

Oxiferm/Enterotubes

These diagnostic tubes are used to identify gram negative bacteria. An oxidase test reagent is placed on a loopfull of isolated 18-24 hour old bacterial colony. If the bacteria produce the enzyme oxidase the reagent will react with the enzyme and the bacterial colony will turn dark purple after a few seconds. These bacteria are considered oxidase positive. They will be inoculated into each compartment of the oxiferm tubes (Hoffmann-LaRoche). This tube helps to identify gram negative oxidase positive bacteria. Most compartments contain a medium which includes a pH indicator. If the bacteria can utilize what is present in the medium the products produced will cause a shift in pH which is indicated by a color change in that compartment. These changes appear approximately 24-48 hours after incubation at 37-38°C.

If the bacterial colony does not change to a purple color when the oxidase reagent is added, these bacteria were inoculated into the Enterotube (Hoffmann-LaRoche). These tubes are used to identify gram negative oxidase negative bacteria. This tube has to be incubated for 24 hours at 37-38°C.

For the above tubes (Oxiferm and Enterotubes) the color changes are noted against a blank tube (not inoculated) as well as for observance of gas production. The results are recorded on coded sheets, the code is found in the manual (Code Book) provided by the

manufacturers and the bacterial genus and species are identified. In some cases there is more than one bacterial species listed for the same code number. In this case, confirmatory tests are performed to eliminate all but one bacterium. (ie. 42°C, sensitivity to certain antibacterial agents, odor, pigmentation, etc.)

For gram positive bacteria the Enterotube were also inoculated but the results were not coded; they were just marked positive or negative for each test. The reason for use of the Enterotubes is the rapid biochemical test results which can be used along with other test results using Bergey's Manual of Determinative Biology to identify the bacterium isolated.

Blood Agar Medium

This is categorized as a differential media. Once a bacterium has been isolated it can be streaked onto the surface of Blood Agar plates and incubated for 24 hours at 37°C. The bacterium should be 18-24 hours old.

If the bacterium produces an enzyme which will lyse the red blood cells (rbc) in the medium, a zone will appear around the bacteria. There are three (3) types of lysis that can occur: (1) hemolysis is the presence of a greenish zone around the bacteria, (2) hemolysis will show a clear zone surrounding the bacteria and, (3) hemolysis is seen when no zone of lysis occurs. Each type of hemolysis will identify a particular species of bacteria if the genus Staphylococcus or Streptococcus is determined (gram positive cocci). It will also help to narrow down the field of other gram positive bacteria.

Dextrose Tryptone Agar (DTA)

This media is used to identify bacteria which can utilize dextrose in the medium. There is a pH indicator (bromcresol purple) which makes the medium purple. If the dextrose is utilized, the metabolic indicators causes a shift in pH which turns the medium yellow which constitutes a positive test. Once the plate is inoculated it must be incubated for 24 hours at 37°C before the color change is noted.

Catalase Test

An 18-24 hour bacterial colony was used to perform this test. A few drops of 3% hydrogen peroxide (H_2O_2) were placed on the colony. If the enzyme catalase is present in the bacterial colony it breaks down the H_2O_2 to release free O_2. Bubbles indicate a positive test.

Slide Coagulase Test

Part of an isolated bacterial colony was mixed with sterile distilled water on a clean glass slide. The slide was observed for self-agglutination for 10-20 seconds. If this did not occur, a small amount of lypholyzed rabbit plasm was added to the bacterial suspension. If clumping occurs, the presence of coagulase enzyme is indicated (positive test).

Phosphatase Test (Gram positive cocci)

One (1) ml of 1% Seitz- filtered phenolphthalein phosphate was added to 100 ml of Nutrient Agar and poured into plates. Plates were inoculated with an 18-24 hour old isolated bacterial colony and

incubated overnight at 37°C. The plates were exposed to ammonia vapors. Colonies of phosphatase positive staphylococci will turn pink due to free phenolpthalein. Staphylococcus aureus is phosphatase positive. Coagulase negative staphylococci and micrococci are usually phosphatase negative.

Oxidase Test

0.01 grams of N,N,N',N'-tetramethyl-p-phenylenediamine is added to 1 ml of distilled water. This oxidase test reagent is placed on Whatman #1 filter paper. The isolated 18-24 hour old bacterial colony is streaked onto the filter paper. The enzyme oxidase, if present, oxidizes the aromatic amine to form colored end products. The oxidation is related to the presence of cytochrome C in the respiratory chain. A purple color after a few seconds indicates a positive test.

All these tests along with shape, size, and arrangement help to identify gram positive bacteria using Bergey's Manual.

Procedure for Petrof-Hauser Counter

Cell depth of 0.02nm NA= 1.3
Ruling is 1/400 sq.mm Neubauer Pattern

Using a 50 microliter capillary tube, 50 microliters of mixed liquor sample was drawn and placed on a Petroff Hauser counting chamber. If the number of bacteria is too large for a fairly accurate count, (number per square should be within 10% of each other) use a 1:10 or a 1:100 dilution.

The number of bacteria was counted in at least 20 squares. Divide the total number bacteria in 20 sq. by 20 to obtain the average per square.

$$\frac{\text{Total bacteria counted} \times 20{,}000{,}000 \times \text{dilution}}{\text{Number of small squares counted}} = \frac{\text{Number of bacteria}}{\text{cm}^3}$$

Figure B-1 shows the Petroff-Hauser bacterial counter and an example of what is seen under the microscope.

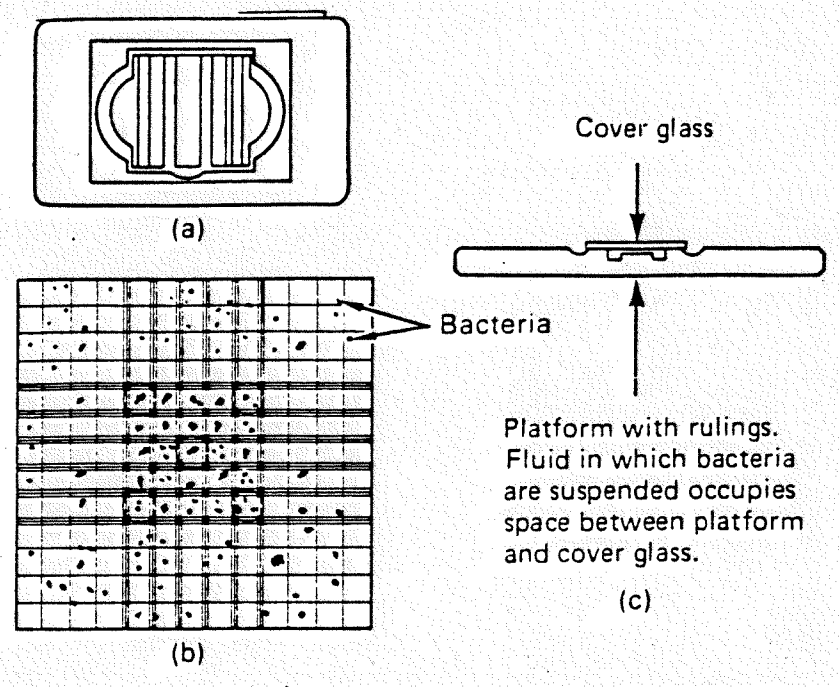
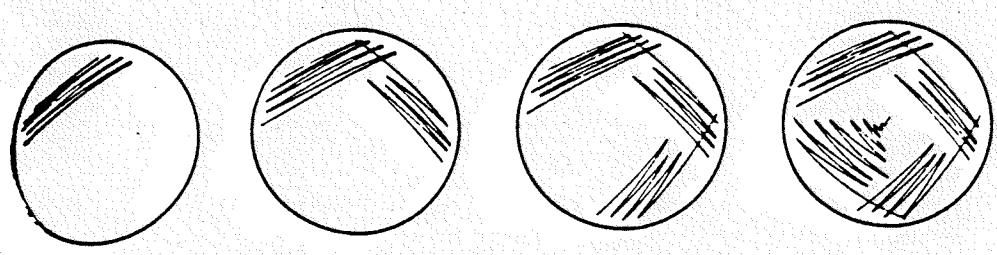
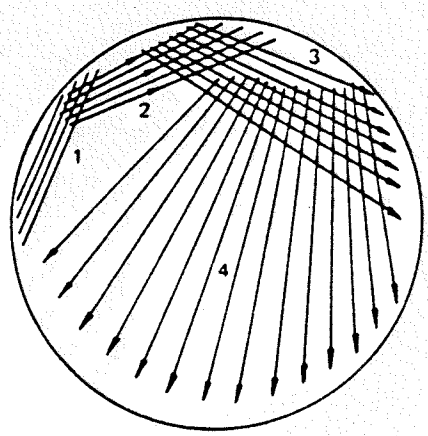


Figure B-1 (a) A Petroff-Hauser bacterial counter. (b) A vertical section view. (c) An enlarged view of the ruled chambers in the center. (31)

Streaking Technique of Agar Plates



The clock-plate method of streaking.



Quadrant Streak Method

a. Spread the organisms over a small area near the edge of the plate. This is **Area 1.** *Apply the loop lightly* to the medium to avoid digging into it.

b. Flame the loop and cool it for 5 seconds.

c. Make five or six streaks from **Area 1** into **Area 2.** Stay near the edge of the plate as shown.

d. Flame the loop again and allow it to cool.

e. Make six or seven streaks from **Area 2** into **Area 3** as shown.

f. Flame the loop again and make as many streaks from **Area 3** into **Area 4** as possible. The remainder of the medium is streaked out as shown.

g. Flame the loop again before putting it down.

Uni-Yeast Tek Plates

Yeast cells which are germ tube negative are incubated into Uni-Yeast tek plates. These plates work on the same idea as the Enterotubes and Oxiferm tubes. The yeast tek plates have 11 wells which contain different sugar media. If the yeast can utilize the sugars the metabolic indicators will cause a shift in pH which is denoted by a color change. Since yeast take longer to grow the plates are observed for six (6) days (noting color change on the day it occurs) and they are incubated at room temperature throughout the six (6) days. The yeast cells are also observed under the microscope to determine which type of reproduction it undergoes. This, along with the biochemical test results are recorded on coded sheets and a code book is used to identify the yeast isolated. Again there may be more than one yeast tested for each code number. Confirmatory tests are used to identify the correct yeast.

Appendix C

Raw Data

Table C-1
Biochemical Test Results for
Gram Negative and Gram Positive Bacteria

Biochemical Test Results for Gram Negative Bacteria

Descrip- / Tests	Gram Stain		Catalase	Coagulase	Oxidase	DTA	Blood Agar	Aerobic Dext	Arginine	N_2 gas	H_2S	Indole	Xylose	Aerob Dext	Glucose	Gas	Lysine	Ornithine	H_2S	Indole	ADON	Lactose	Arabinose	Sorbitol	Dulcitol	P.A.	Urea	Citrate
F-Scr H-10	⊖	cocci	-		+								+	+	V						V						+	+
Pc Scr H-12	⊖	rods/cocci	-		-								+	+	+						+						+	+
F Smy H-10	⊖	cocci	+		+								+	+	+													
Pc Smy H-12	⊖	rods	+		V								+	+	+						+							
F Ly/c H-11	⊖	rods	+		-																						+	+
Pc y H-10	⊖	long chains rods	+		-																						+	+
Pc L y H-11	⊖	rods	+		-																+							
Pc Cr Tr H-10	⊖	rods								Y			BL		V													+
F Trans H-12	⊖	rods			-																							+
F Sy H-11	⊖	rods																										
F wh/cr H-12	⊖							+					+	+	V							+	+					
Pc Smcr H-12	⊖														Y													+
Cell Tydt H-12	⊖	short rods													+				+			+	+				+	+
F wh H-12	⊖	rods chains			-																		+					Y
F lawh H-11	⊖	short rods			+																		V					Y
F med cr H-11	⊖	cocci			-																							Y
F furru wh H-12	⊖	short rods			+									BL	BL													
Pc wh Biob H-10	⊖	cocci			+								BL		+											V	+	-
Pc er tydot H-11	⊖	cocci			-			+	+				+	+	+							+	+	+		+	-	+
Pc Y/lo dt H-11	⊖	cells			-			+	+				+	+	+							+	+	+		+	-	+
F Sm wh H-11	⊖	rods			-																							NG
F sm y H-11	⊖	rods cocci			-																							NG

H- fresh mixed liquor
P- phenol acclimated (100 ppm)
OP- 2-chlorophenol acclimated (20 ppm)
O- 2-chlorophenol shock-load
BL- basic products produced
Y- yellow color
V- variable test results
NG- no growth observed

Table C-1 (continued)

Biochemical Test Results for Gram Negative Bacteria

Tests / Descrip-	Gram Stain			Catalase	Succinate	Oxidase	OTA	Mixed Acid	Arginine Anaerobic Dehydrase	Arginine	N_2 Gas	H_2S	Indole	Xylose	Anaerobic Dehydrase	Glucose	Sucrose	Lysine	Ornithine	H_2S	Indole	Urea	Lactose	Arabinose	Sorbitol	Dulcitol	PA	Urea	Citrate
F Sm Trickle OP10	⊖	rods				+					+					V													
PC Lg Blob OP12	⊖	cocci				V			+		V																		
PC Y/Cr OP10	⊖	rods				-																							
PC Cr OP11	⊖	rods				+					+					+							+			+			+
PC Wh Cr OP10										+		+		BL	BL	+				+							+	+	
F Wh OP12										+		+		BL	BL	+				+							+	+	
F Tr Cr OP11																													
PC Wh Cr OP11										+		+		BL	BL	+				+							+	+	V
PC Wh OP12										+		+		BL	BL	+				+		+					+	+	V
F Tr Cr OP10	⊖	rods				-								BL	BL														
F Tr Cr OP10	⊖	rods				-								V	+													+	+
Wht Y/Cr OP10	⊖	rods				+								V	+													+	+
PC Y OP10	⊖	small rods				-										+							+	+					
F Y/Cr OP10	⊖	long rods				+									BL														
PC Shiny Gr OP10	⊖	rods				+										+							+	+					
PC Cr OP10	⊖	rods				+								V	+													+	+
PC Sm Y OP12	⊖	cocci		+		+		B																					
F Shiny Wh OP11	⊖	rods				+			+					BL	BL													+	+
PC11 Sm Tr OP11	⊖	cocci				+			+					BL	BL													+	+
PC Cr OP12	⊖	rods				+			+					BL	BL													+	+
PC Sm Wh OP12	V	rods				-								BL	BL														+
PC Tr OP11	⊖	rods				+			+					BL	BA													+	+

H- fresh mixed liquor
P- phenol acclimated (100 ppm)
OP- 2-chlorophenol acclimated (20 ppm)
O- 2-chlorophenol shock-load
Bl- basic products produced
Y- yellow color
V- variable test results
NG- no growth observed

Table C-1 (continued)

Table C-1 (continued)

Biochemical Test Results for Gram Negative Bacteria

Description / Tests	Gram stain	Shape	Catalase	Sugars	Oxidase	DTA	Blood Agar	Arginine Anaerobic Dextrose	N_2 gas	H_2S	Indole	Xylose	Glucose Aromatic Dextrose	Glucose	Gas	Lysine	Ornithine	H_2S	Indole	ONPG	Lactose	Arabinose	Sorbitol	Mannitol	PA	Urea	Citrate
H: CV 0-11					+							+	+	+								+			V	+	+
V: Thin dust 0-10					-							+	+	+								+				+	+
F: Wh 0-12					+							+	+	+								+				+	+
OP: Thick Cell Lab 0-12			+		-							+		+							+	+				-	+
F: Tr Wh 0-11			-		+							+	+	+							+	+				+	+
E: Orange 0-8			-		-																						
F: Yellow 0-12			+		V																						

H- fresh mixed liquor
P- phenol acclimated (100 ppm)
OP- 2-chlorophenol acclimated (20 ppm)
O- 2-chlorophenol shock-load
V- variable test results
Bl- basic products produced
NG- no growth observed

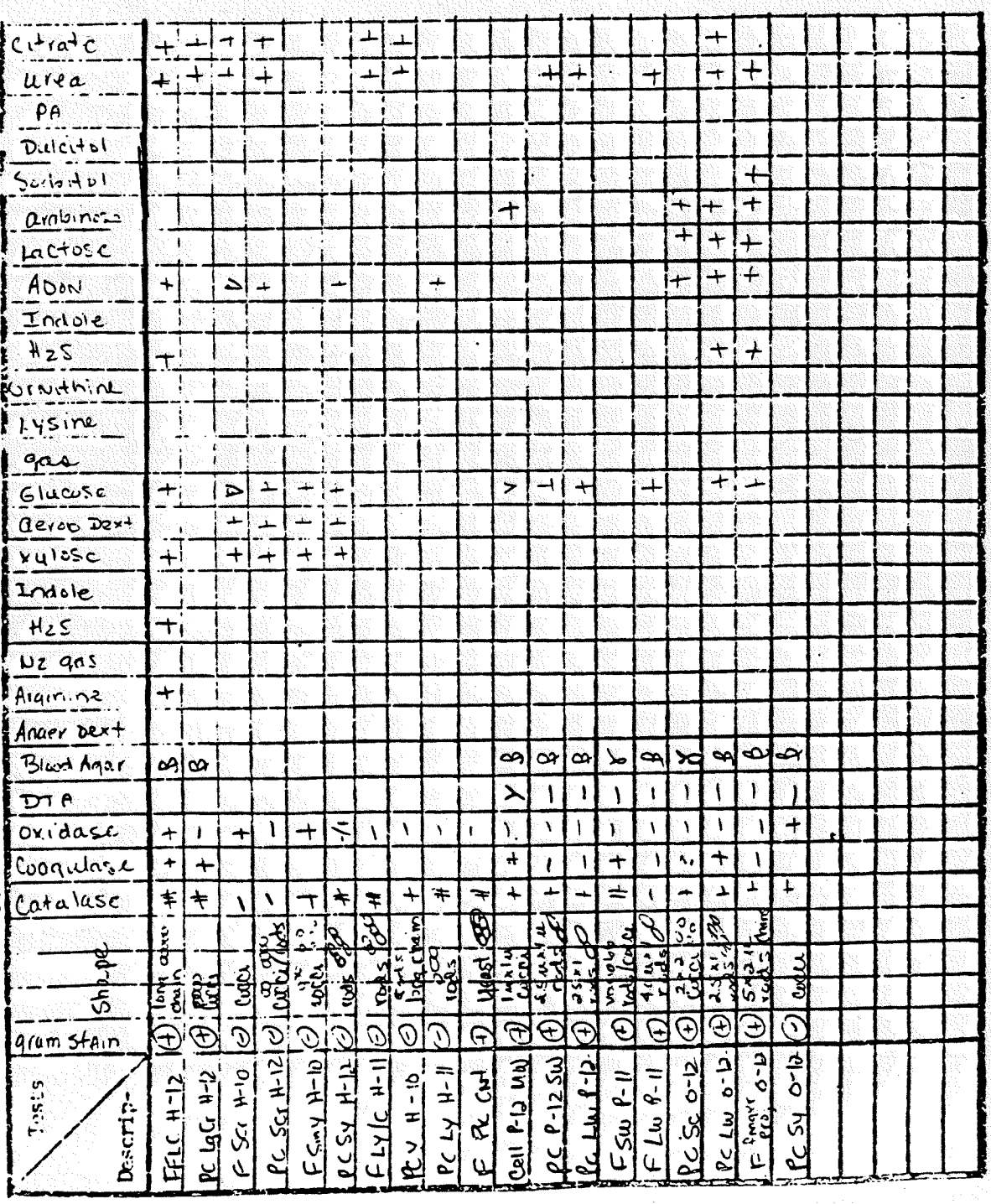
Biochemical Test Results for Gram Positive Bacteria

Tests / Descrip-	Gram stain	Shape	Catalase	Coagulase	Oxidase	DNA	Blood Agar	Arginine Dihydrolase	N_2 gas	H_2S	Indole	Xylose	Fermentative Dextrose	Glucose	Gas	Lysine	Urease	H_2S	Indole	Mannose	Lactose	Arabinose	Salicin	Dulcitol	PA	Urea	Citrate
F LC H-12	⊕	long chains rods	+	+	+		β	+		+		+		+				+		+						+	+
Pc LC H-12	⊕	coccoid cocci	+	+	−		β																			+	+
Pc LW H-11	⊕	long rods			+									+												+	+
Pc cr H-12	⊕	rods			−			+		V		BL	+	+												+	+
Cell MW P12	⊕	1μ×1μ cocci	+	+	−	Y	β							V								+				+	
Pc SW P12	⊕	.25μ×1μ rods	+	−	−	−	β							+												+	
Pc LW P12	⊕	.25×1μ rods	+	−	−	−	β							+												+	
F SW P-11	⊕	variable rods/cocci	+	+	−	−																					
F LW P-11	⊕	1μ×1μ rods	−	−	−	−	β							+												+	
Pc slimy P-11	⊕V	short cocci rods 1μ	+	−										+						+	+				+		
Pc LW P-12	⊕	long rods	+	+				+				BL	BL	+				V							+	+	Y
F MW P-11	⊕V	long curved rods	−	−				+				BL	BL	+											+	+	+
Pc Ser OP12	⊕	simple rods/cocci			−									+												V	
F transparent OP10	⊕	rods			+																						
Pc Sc O-12	⊕	2μ×2μ cocci	+	V	−	−	γ													+	+	+					
Pc LW O-12	⊕	.5×1 rods	+	+	−	−	γ							+				+		+	+	+					
F finger print O-12	⊕	5μ×2μ rods chains	+	−	−	−	β							+				+		+	+	+	+			+	
Pc cr shiny O-12	⊕V	rods		+		+	−	β		+					+	+											+	+
F SY O-11	⊕	rods				+			+			V			+	+											+	+
Pc cr O-12	⊕	cocci		+		−											+											

H- fresh mixed liquor
P- phenol acclimated (100 ppm)
OP- 2-chlorophenol acclimated (20 ppm)
O- 2-chlorophenol shock-load
Bl- basic products produced
Y- yellow color
V-variable test results
NG- no growth observed

Table C-1 (continued)

Table C-2
Characterization Test Results for
Bacteria and Yeast, Runs 1-4



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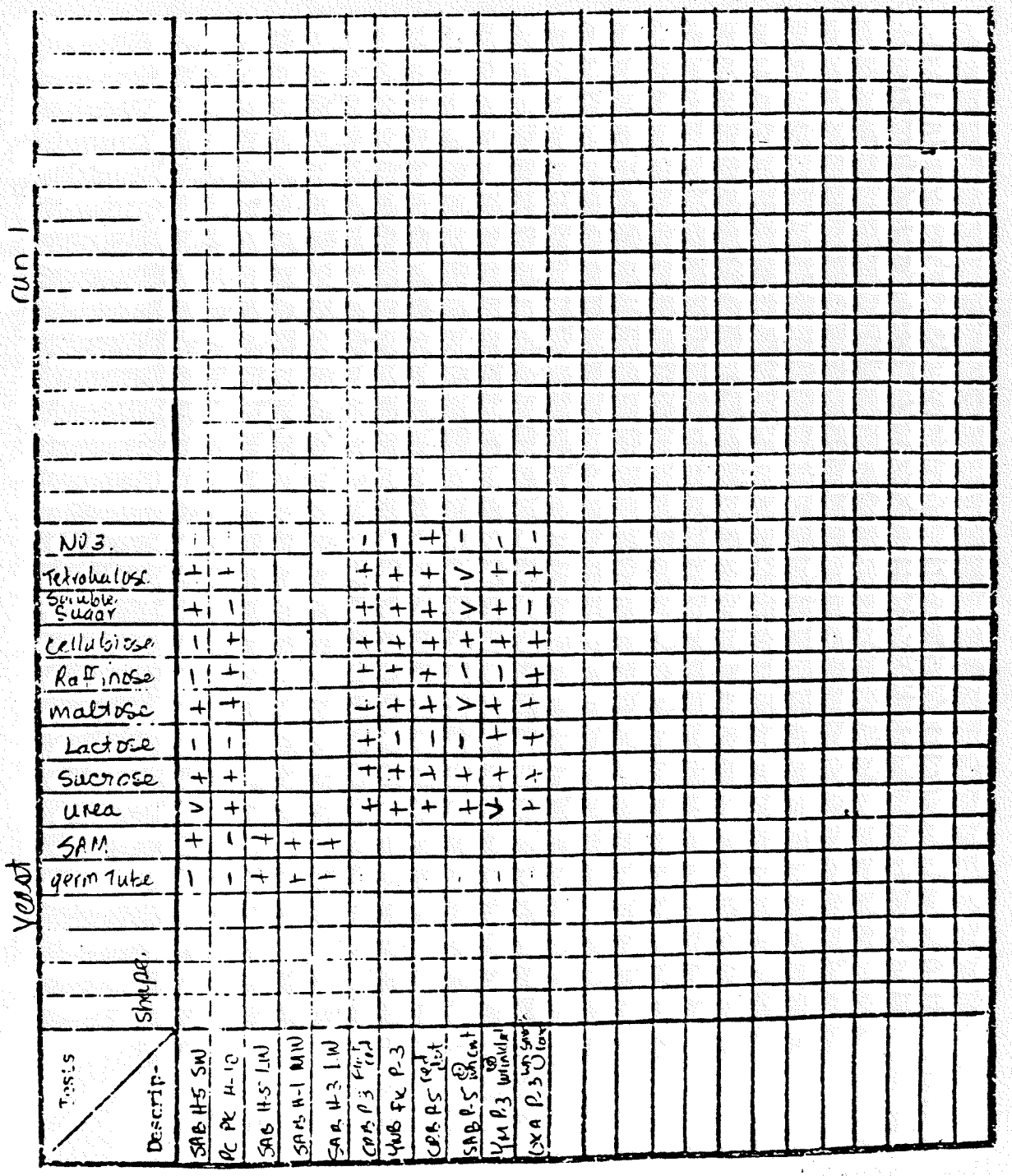
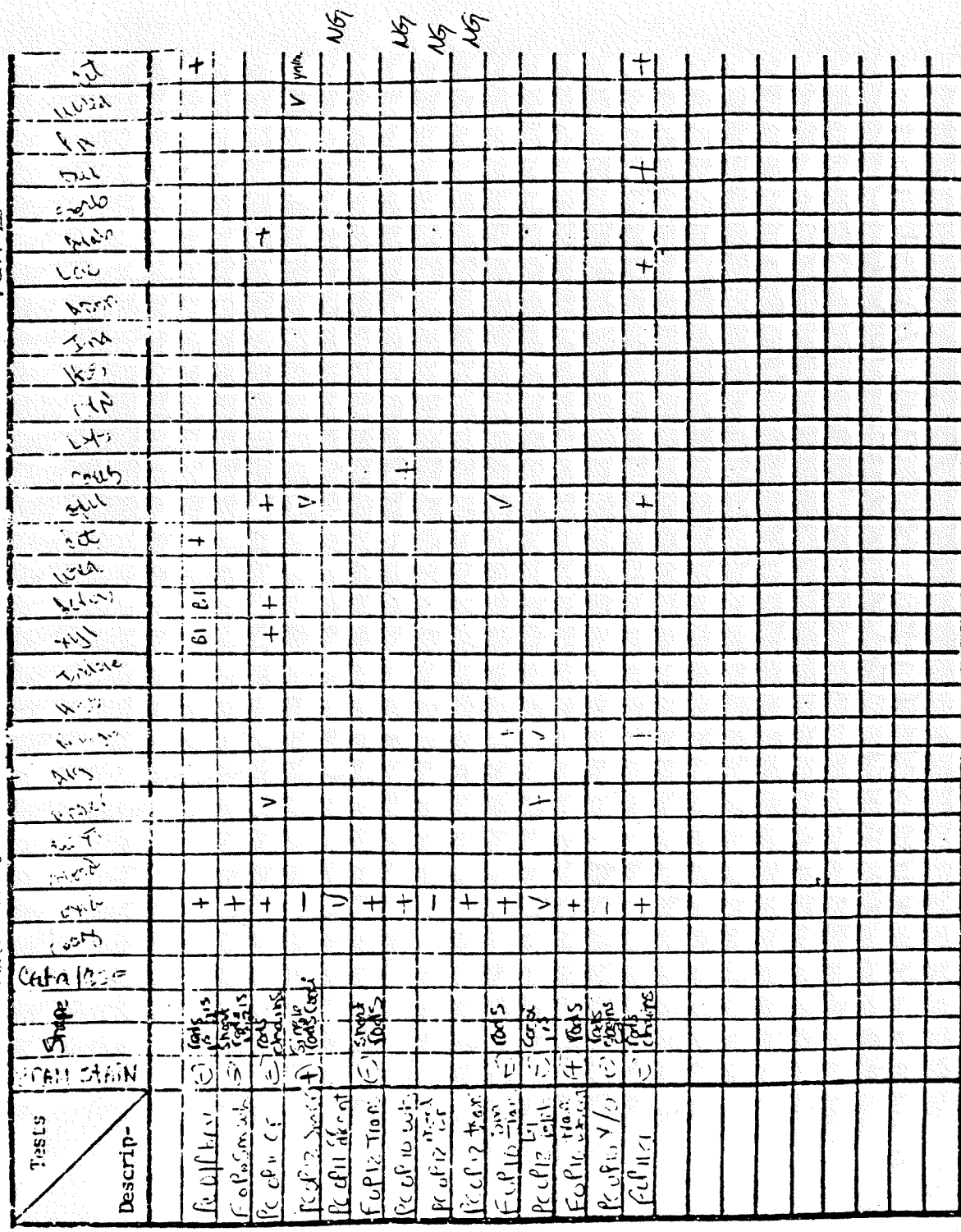


Table C-2 (continued)

Raw Data — run 2

Tests / Descrip-	Gram Stain	Shape	Catalase	Coag	Oxidase	Bl ora	Cert DTA	Amgd Dextrose	Arg	N2 gas	H2S	Indole	Xylose	Acr dext	Urea	Citrate	Glucose	gas	Lys	Orn	H2S	Indole	Adon	Lac	Arab	Sorb	Dulcitol	PA	Urea	Citrate
PCH-10 cr TR	⊖	rods								V			Bl			⊥	V													+
FH-12 tran.	⊖	rods			−																									+
FH-11 SY	⊖	rods																												
FH-11 Ty wh	⊕	yeast							+		+		Bl	Bl	+	+	+				+								+	+
FH12 wh/CR	⊖	rods						+					+	+			V							+	+					
FH-11 PK	⊕	yeast			−			+					+	+	+	+	V												+	+
FH-11 wh Ty dt	⊕	yeast																												
PCH-12 sm r	⊖	rods															V													+
PCoH 11-12 Ty dt	⊖	short rods															+				+			+	+				+	+
PCH-11 LW	⊕	long rods															+												+	+
FP-11 Sm wh	⊕	yeast			−																									
FP11 PK	+/−	yeast rods			−			+					+	+	+	+														
FP11 TR	⊖	rods			−				+				+	+	−	+														
FP-11 Y	⊖	rods			+				+				+	+	−	+														
PCO-12 cr Shiny	+/−	rods			+				+				+	+	+	+														
FO-11 Shiny wh	−/+	rods			+			+					Bl	Bl	+	+														
FCoH O-11 sm TR	⊖	circ rods			+			+					Bl	Bl	+	⊥														
PCO-12 cr TR	⊖	rods			+			+					Bl	Pl	+	+														
PCO-12 Y	+/−	rods			+			+																						
PCO12 Sm wh	−/+	rods											Bl	Bl		+														
PCO12 Orange	+/−	yeasts																												
FO11 SY	⊕	rods			+			+			V		⊥	+	+	+														
FPCO11 TT	⊖	rods			+			+					Bl	Bl	+	+														

Table C-2 (continued)



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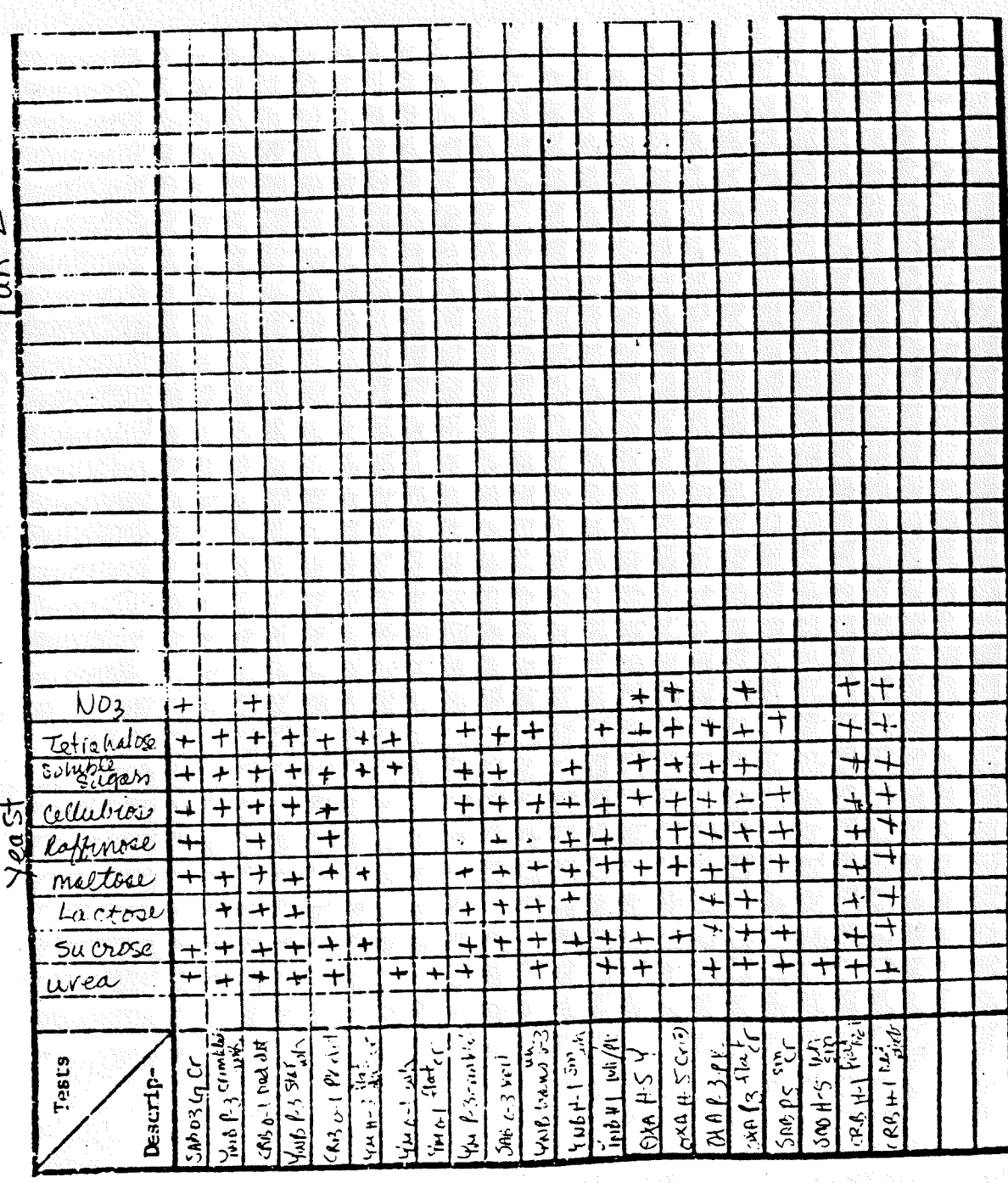


Table C-2 (continued)

Raw Data — run 3

Tests / Descrip-	Gram stain	Shape		Catal	Coag	Oxidase	Dext ferm	Blood	Anaer dext	Arg	No gas	H2S	Indole	Xylose	Aero dext	Bile	Gas	Lyse	Orn	H2S	Mot	NO3/N	Lac	Escul	Sorb	Dul	PA	Urea	Citrate	Cong
FH-12 wh	⊖	rods chain 1's				−																		V					Y	
FH 11 Lg wh	⊖	cocci small rods				+																		V					Y	
FH-11 cr med	⊖	cocci				−																							Y	
FH-12 fuzzy wh	⊖	short rods 2's 1's				+								1	Bl	Bl														
PC H-10 wh Dot	⊖	cocci				+								Bl		+											V	*	−	
PC H-11 cr TV dot	⊖	cocci 1's				−			+	+	−	−	−	+	+	+							+	+	+		+	−	+	
Pc H-11 y/o dot	⊖	cocci diplo				−			+	+				+	+	+							+	+	+		+	−	+	
PC H-12 cr	⊕	cocci 4's				−				+		V		Bl	+	+											+	+	+	
FH-11 Sm wh	⊖	rods				−																		V					Y	
FH-10 wh dot	⊕	yeast				−																								
FH-10 Y dot	⊖	yeast long chains				+																								
FH-11 Sm y	⊖	rods 2's				−																								
Pc P-12 wh	⊖	cocci				−								+	+													+	+	
Pc P12 cr	⊖	thin rods 2's				+								+	+									+			V	+	+	
FP-11 Tr wh	⊖	short rods 2's				+								+	+									+			V	+	+	
Pc P12 Y	⊖	long short rods				+								1	Bl															
Pc o-11 cr	⊖	rods chains				+								+	+									+			V	+	+	
FO-10 Tr wh dot	⊖	rods 2's chains				−								+	+									+			V	+	+	
FO-12 wh Tr	⊖	short rods				+								+	+													+	+	

Table C-2 (continued)

run 3

Tests / Descrip-	Gram Stain	Shape	Cat.	Coag	Oxid	dext ferm	Blood	Amm	Arg	N2 gas	H2S	Indole	K/I	Aerob	Glu	Gas	Mls	ONP	Mis	Ind	Adon	Lac	Aerob	Sorb	Dul	PA	Urea	Citrat	
* PCOP10 wher	(+)	rods							+		+		B1	B1	+				+							+	+	−	
FOP12 wh	(+)	rods							+		+		B1	B1	+				+							+	+	−	
FOP10TR cr	(−)																												
PCOP11 wher	(+)	rods							+		+		B1	B1	+				+							+	+	v+	
PCOP12 wh	(+)	rods							+		+		B1	B1	+	−			+			?				+	+	v	

* data was thrown out because levels of toxicant were not measured during this portion of the experiment.

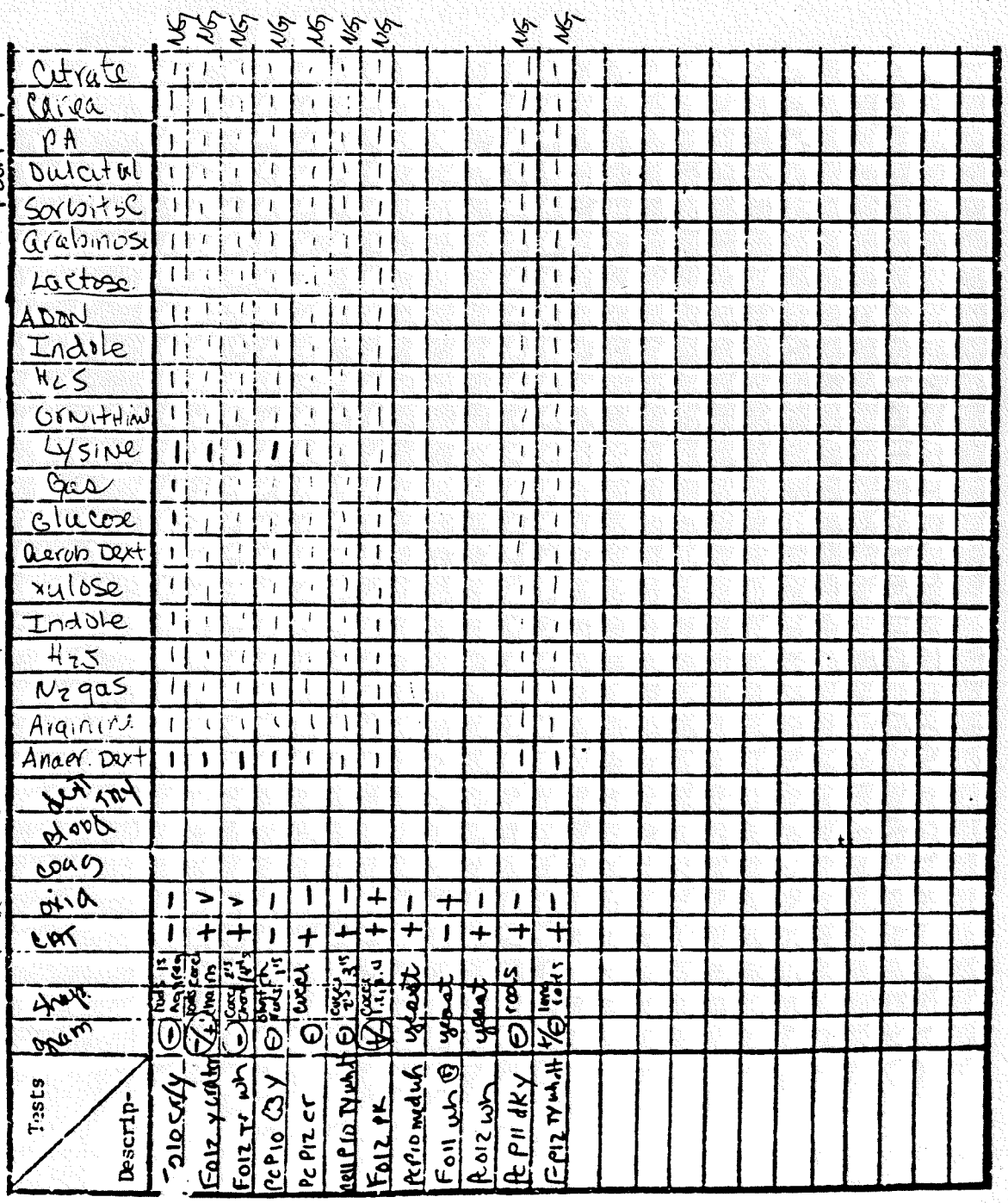
Table C-2 (continued)

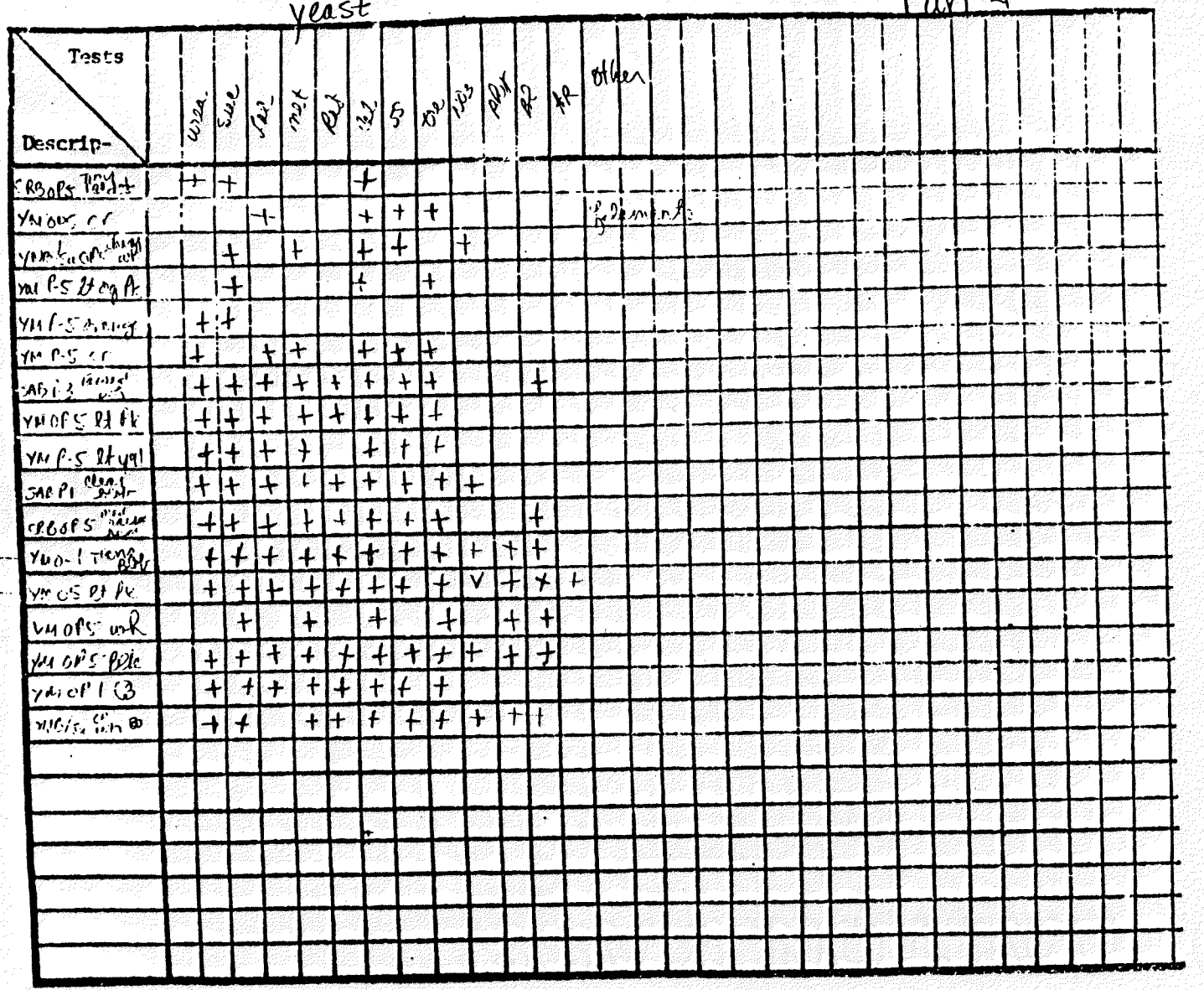
Raw Data — run 4

Tests / Descrip-	Gram Stain	Shape		Catalase	Oxidase	Coag	Blood	Dext Try	Malt Dext	AROS	Nitrogs	H_2S	Indole	Xyl	Aerob Dext	Urea	Citrate	Glucose	ogo	Lys	Orn	H_2S	Indol	Adon	Lac	Arab	Sorb	Dul	PA	Urea	Lit
													−										−								
FP11 SmTr wh	(−)	rods short		+	−				−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+	−	−	−	+	+
FP10 med wh	(−)	short ch rods		+	−				−	+	−	+	−	−	−	+	+	+	−	−	−	+	−	−	+	−	−	−	+	+	+
PcP11 medy	(−/+)	cocci		+	−				+	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−
FP12 (B)	(+)	yeast		+	−				−	−	−	−	−	V	V	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−
Cell D12 TY wh dot	(−)	rods ch cocci		+	−			−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+	+	−	−	−	−	−
FP11 Tran y	(−)	rods		−	−				−	−	−	−	−	+	+	V	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+
PcP11 Smy	(+/−)	short rods cocci 1's		+	−				−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	+	+	−	−	−	−	+	−
PcP12 lg wh	(+)	rods long		+	+				−	+	−	−	−	Bl	Bl	+	+	+	−			V	−	−	−	−	−	−	+	+	V
FD-11 Tr wh	(−)	rods 2's 1's		−	+				−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+
FP-10 Try	(−)	rods		−	−				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
PcD-12 cr	(+)	cocci		+	−				−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
PcP12 medy	(−)	cocci 2's		+	−				−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	−	−	+	−
PcD-11 medy	(+/−)	yeast		+	−				−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−
FP11 ma wh	(+/−)	long ch rods		−	−				−	+	−	−	−	Bl	Bl	+	−	+	−	−	V	−	−	−					+	+	+
PcP-10 med TR	(−)	rods 1's short ch		−	+				−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
PcP10 medy		yeast		+	−				−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	V	−
FP11 Smy	(−)	short ch cocci		+	−				−	−	−	−	−	−	−	+	V	−	−	−	−	−	−	−	−	−	−	−	−	+	−
PcP12 cl (B)		yeast		+	+				−	−	−	−	−	V	V	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−

Table C-2 (continued)

Table C-2(continued)




yeast
run 4
Tests
Descrip-
Other

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