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Abstract

Title of Thesis: **Characterization of Microorganisms After Feeding With Phenolic Compounds: Comparing A Domestic to an Industrial Sludge**

Dianne Adamowitz, Master of Science, 1986

Thesis directed by: Associate Professor, Dr. G. Lewandowski

This study examined the effects of phenolic compounds on the populations in mixed liquors obtained from two very different treatment plants: the Passaic Valley Sewerage Commissioners plant in Newark, and the Livingston, NJ municipal treatment plant. The dominant organisms were identified initially and after ten days exposure to phenol (100 ppm), and either 2-chlorophenol (20 ppm) or 2,6-dichlorophenol (10 ppm) in aerated batch reactors.

Results confirmed the remarkable similarity between the two mixed liquor populations, in spite of the significant differences in the operation of the two treatment plants.

Characterization of Microorganisms After Feeding With Phenolic Compounds: Comparing A Domestic Sludge to an Industrial Sludge

by
Dianne Adamowitz

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 Master of Science in Environmental Science/Toxicology option
1986

Approval Sheet

Title: Characterization of Microorganisms After Feeding With Phenolic Compounda: Comparing A Domestic Sludge to an Industrial Sludge

Name: Dianne Adamowitz
Master of Science, 1986

Thesis and Abstract Approved:

Dr. Gordon A. Lewandowski
Associate Professor
Department of Chemical Engineering and Chemistry

5/15/86
Date

Dr. Joseph W. Bozzelli
Professor
Department of Chemical Engineering and Chemistry

May 16 86
Date

Dr. John E. McCormick
Professor
Department of Chemical Engineering and Chemistry

5/16/86
Date

VITA

Name: Dianne Adamowitz

Permanent Address:

Degree and Date to be Conferred: M.S., 1986

Date of Birth:

Place of Birth:

Secondary Education: Hillside High School, 1979

Collegiate Institutions	Dates	Degree	Date of Degree
Union County College	9/79 - 5/81	A.A.	May 1981
Cook College/Rutgers University	9/81 - 12/83	B.S.	January 1984
New Jersey Institute of Technology	9/84 - 5/86	M.S.	May 1986

Major: Environmental Science/Toxicology Option

DEDICATION

To my grandmother, Adelaida Santangelo

Acknowledgements

I would like to thank Dr. Lewandowski for his faith in me. I would also like to thank my family for all their love, support, and patience.

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Introduction

In addition to the treatment of domestic sewage at wastewater treatment facilities, many industrial wastes are also being treated. Wastes that enter the facility are confronted by an established open ecosystem. Organisms present have been introduced from such sources as the surrounding soils, waters, from decaying habitats, and from the sewage itself. The wastes are utilized by the organisms (primarily bacteria) as a source of food and energy. The result is that organic molecules entering the treatment plant are partially or completely degraded, so that the receiving stream is not overloaded with growth inducing (and hence oxygen depleting) nutrients.

Though many organisms enter the facility, the ones which will persist will depend upon the nature of the waste and the interactions among the various species of bacteria, yeast, fungi, protozoa, rotifers, and other organisms present. Each organism has a specific niche that helps balance this complex environment.

According to Benedict and Carlson (1971) the dominant bacterial genera present in sewage include, Acinetobacter, Alcaligenes, Caulobacter, Flavobacterium, Microbacterium, Pseudomonas, and Sphaerotilus, while the dominant yeast is Debaromyces. Pathogenic bacteria have also been discovered, including Shigella, Klebsiella pneumoniae, and

Staphylococcus aureus (Dudley 1980). Many of the species present include members of the family Enterobacteriaceae and other gram negative, oxidative positive enteric bacteria (figure 1).

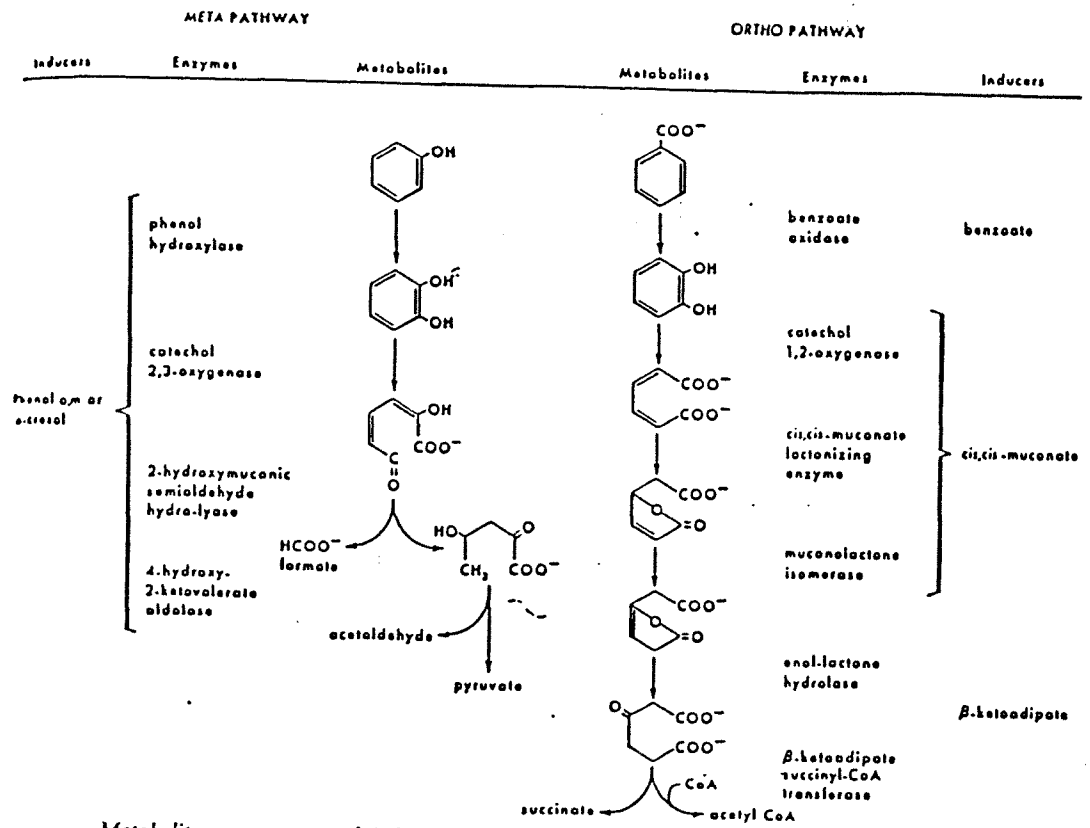
Some of the industrial compounds which provide sustenance to the microorganisms include the phenolic compounds since their sources are so widespread. The origin of the phenolics include coal and wood distillaries, road tars, petroleum refining, chemical and plastics manufacturing, wood preservatives, livestock dips, coal and oil deposits, and plant material decay.

The effects associated with phenolic compounds include direct toxicity to aquatic life, imparting bad taste and odors to edible fish and shellfish,and lowering of the dissolved oxygen content in streams. Phenol containing waters when chlorinated may produce toxic polychlorinated phenols. As a result the Environmental Protection Agency has set a phenol standard of < 1 ppb in surface waters.

It is therefore useful to know how the microorganisms in a wastewater treatment plant will be affected by the presence of phenol. Table 1 shows some examples of phenol degrading organisms, and Table 2 is an indication of relative degradation rates for phenolic compounds.

It has been found that soil microbes such as Pseudomonads have developed the capability of degrading a wide range of complex naturally occurring aromatic and

Meta and Ortho Pathways (figure 1)
(Feist et.a., 1969)



Metabolites, enzymes, and inducers of the ortho and meta pathways in P. putida. The regulation of the ortho pathway was described by Ornston (18). Enzymes, the names of which are enclosed by brackets, share a common inducer.

Table 1

Frequency of occurrence and distribution of bacteria degrading phenolic compounds (Tabak, et. al.,1964)

Classes of compounds degraded	Sources of cultures	Generic grouping of culture*			
		Pseudomonas	*Achromobacter*	*Flavobacterium*	*Xanthomonas*
Nitrophenols	Garden soil, compost, and river mud	23	3	14	3
Chlorophenols	Sediment from petroleum refinery wastes lagoon	37	3	3	0
Cresols	Sediment from petroleum refinery wastes lagoon, garden soil, and compost	48	8	4	0
Phenol	Sediment from petroleum refinery wastes lagoon	12	1	1	0
Alkyl phenols	Garden soil, compost, and refinery lagoon sediment	8	0	1	1
Aryl phenols	Garden soil, compost, and refinery lagoon sediment	11	4	1	0
Hydroxy phenols	Garden soil, compost, and refinery lagoon sediment	13	6	1	0
Per cent occurrence†		73.8	12.1	12.1	1.93

* Figures represent number of cultures, each having different biochemical or other characteristics, isolated from media in which a compound in the class indicated served as the sole source of carbon.

† Per cent of a total of 200 isolations falling in the respective genera indicated.

Table 2

Time required for bacteria to utilize 95% of parent substrate

Compounds degraded in*		
1 to 2 days	3 to 6 days	7 to 10 days
Phenol	*o*-Nitrophenol	2,4-Dinitrophenol
	m-Nitrophenol	
Catechol	*p*-Nitrophenol	2,6-Dimethylphenol, 200 ppm
Resorcinol	2,4,6-Trinitrophenol, 250 ppm	2,4-Dichlorophenol, 200 ppm
Quinol	2-Chloro-4-nitrophenol	2,4,6-Trichlorophenol
Phloroglucinol	2,6-Dichloro-4-nitrophenol	
o-Cresol	*m*-Chlorophenol, 150 ppm	
m-Cresol	*p*-Chlorophenol	
p-Cresol	*o*-Phenylphenol, 100 ppm	
	Thymol, 150 ppm	

* Initial concentration was 300 ppm unless indicated otherwise.

(Tabak, et.al., 1964)

aliphatic compounds (Don and Pemberton,1981). It was found that those organisms which were able to degrade these compounds, possessed plasmids which encoded part of, or the entire, degradative pathway. Feist and Hegeman (1969) have determined that a number of aromatic compounds which support the growth of fluorescent pseudomonads (P. aeruginosa, P. putida, and P. fluorescens) are catabolized via the common diphenolic intermediate, catechol (see figure 1). The catechol precursor induces catechol 1,2-oxygenase and associated enzymes of the B-ketoadipate pathway (ortho cleavage) (although it has been found in some strains that catechol can be degraded by an alternate inducible pathway using a catechol 2,3-oxygenase). Many of the strains which are able to decompose compounds via the meta-cleavage pathway also possess the genetic ability to degrade by the ortho pathway (Feist and Hegeman 1969). The pathway is determined by the precursor molecule of the catechol upon which the organism is grown. Using cell extracts of a mutant strain of Pseudomonas putida (strain 144 Pseudomonas U), which had been grown on phenol, indicated that degradation took place via the meta-pathway. However, extracts of the same strain grown on benzoate contained only enzymes of the ortho-pathway. When catechol itself was used, only ortho enzymes were found. Using both catechol and phenol as feed, enzymes of both pathways were found.

Catechol 2,3-oxygenase (meta) is induced by the primary substrate of the pathway, while catechol 1,2-oxygenase (ortho) is induced by the product of its action. The two catechol cleavage enzymes differ in specificity; Induction of meta-pathway occurs by cresol or phyenol is under less specific control than ortho-pathway induction which occurs by a product of one of the pathway enzymes. Low specificity of the meta-pathway implies that during its evolution its function was to catabolize a variety of naturally occurring aromatic compounds, while the ortho-cleavage is specific to catechol itself.

Another variation on the meta pathway has been demonstrated by Bayly and Wigmore (1973) for Pseudomonas putida (see figure 2). The product of the pathway, pyruvate, then leads into the citric acid cycle for further biotransformation. The addition of NAD(+) - dependent aldehyde dehydrogenase to cell extracts of phenol-induced P. putida resulted in an increased rate of disappearance of 2-hydroxymuconic semialdehyde. By the use of mutant strains of P. putida U, the products of the ring fission were studied. Table 3 shows the mutant strains derived from wild-type Pseudomonas putida (PsU-O) which were able to revert and use phenol and/or cresol as its carbon and energy source. About 3% of the apparent revertants isolated on phenol could grow on each of the cresols as a sole carbon source. The remainder were partial revertants with an

META CLEAVAGE PATHWAY (figure 2)
(Bayly, et.al., 1973)

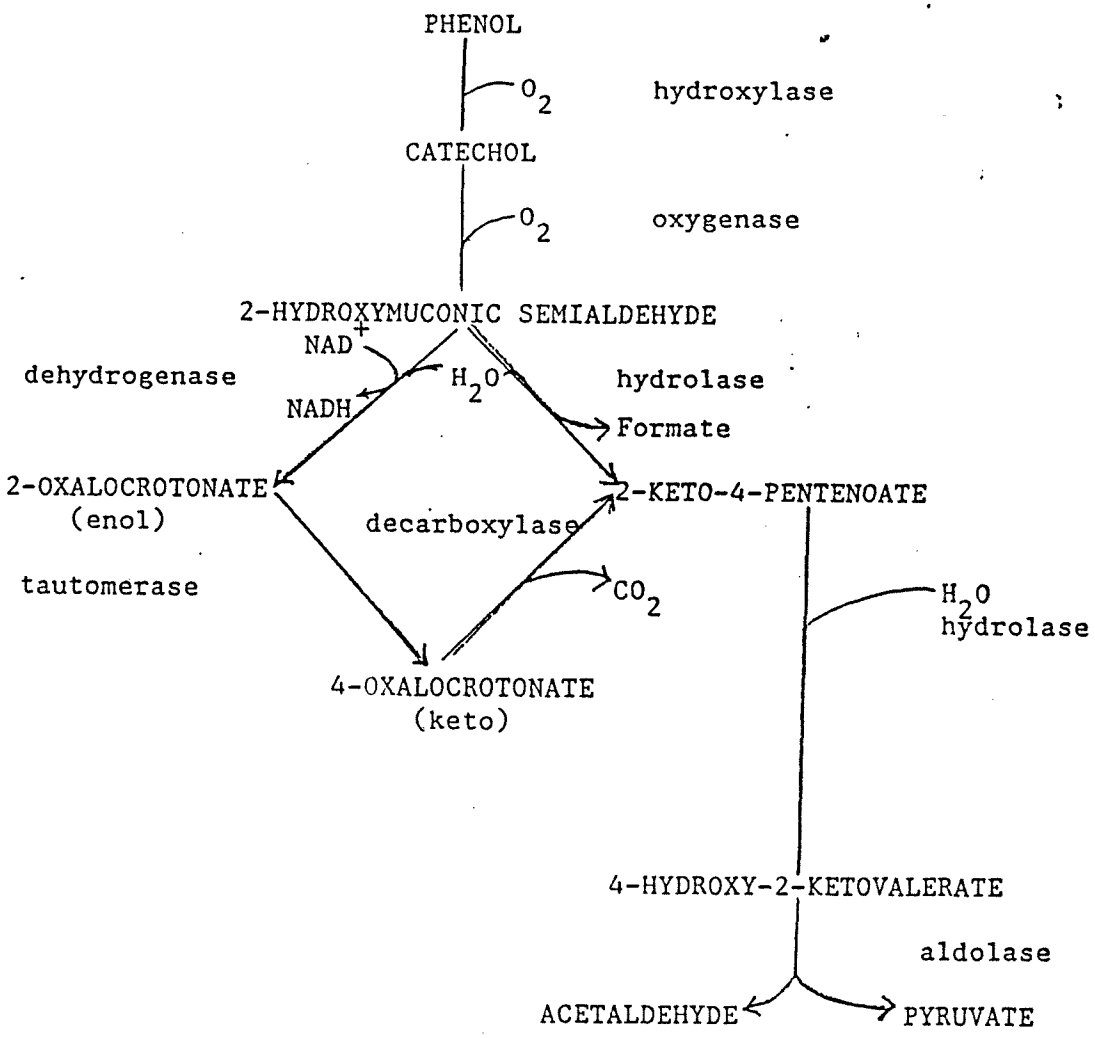


Table 3

Mutant strains derived from wild-type PsU-O

(Bayly, et.al., 1973)

Mutant strain	Mutagen	Selection and characteristics	Immediate parent
PsU-5	EMS	Unable to use phenol as a sole carbon source.	PsU-O
PsU-D1	EMS	Absence of catechol 2,3-oxygenase when grown on phenol; unable to use any cresol as a sole carbon source.	PsU-O
PsU-5-R5	None	Able to grow on phenol; on replica plating able to use each cresol as a sole carbon source.	PsU-5
PsU-5-R11	None	Able to grow on phenol; on replica plating unable to use any cresol as a sole carbon source.	PsU-5
PsU-5-R21	None	Able to grow on *o*-cresol; on replica plating able to grow on *m*-cresol but not phenol or *p*-cresol.	PsU-5

extragenic suppressor mutation, causing the absence of catechol 2,3-oxygenase.As a result, phenol was degraded by the alternate ortho-path. Cell extracts when grown on phenol were shown to contain high amounts of catechol 1,2-oxygenase and cis,cis-muconate lactonizing enzyme (ortho-pathway), but no detectable levels of catechol 2,3-oxygenase, phenol hydrolase, or NAD(+) -dependent aldehyde dehydrogenase. Table 4 shows the enzyme activities of certain mutants and wild-type strain PsU-O. Absence of detectable levels of NAD(+) -dependent aldehyde dehydrogenase in PsU-5 accounts for high levels of ring fission products from phenol and cresol decomposition. The ability of mutants to regain both of these activities spontaneously occurs at a frequency of 2 out of 10 cells plated. Partial revertant frequencies are demonstrated in Table 5. Thus by the use of mutant strains, it was shown that phenol degradation occurs in three ways: (1) conversion to catechol, then (by meta-fission) to 2-hydroxymuconic semialdehyde, which is converted to 4-oxalocrotonate;, (2) same method up to hydroxymuconic semialdehyde which is metabolized to 2-keto-4-pentenoate; and (3) by conversion to catechol which then undergoes ortho-fission of the aromatic nucleus.

Wong and Dunn (1975) have found that a transmissible toluate-utilizing plasmid encodes for the

Table 4

Specific activities[a] of enzymes of the ortho and meta fission pathways when selected strains were grown to stationary phase on phenol as the sole source of carbon (Bayly, et.al, 1973)

Strain	Carbon source	Catechol 1,2-oxygenase	Muconate lactonizing enzyme	Catechol 2,3-oxygenase	Hydrolase	Hydrolase and dehydrogenase
PsU-O	Phenol	<0.001	<0.001	0.177	0.006	0.036
	Benzoate[b]	0.065	0.023	<0.001	<0.001	<0.001
PsU-D1	Phenol	0.047	0.017	<0.001	0.004	0.019
PsU-5-R11	Phenol	0.047	0.015	<0.001	<0.001	<0.001
PsU-5-R21	Phenol	<0.001	<0.001	0.141	0.006	0.006

[a] Expressed as micromoles of substrate used (or product formed) per minute per milligram of protein.
[b] Included to provide a comparison for strains using the *ortho* fission pathway.

Table 5

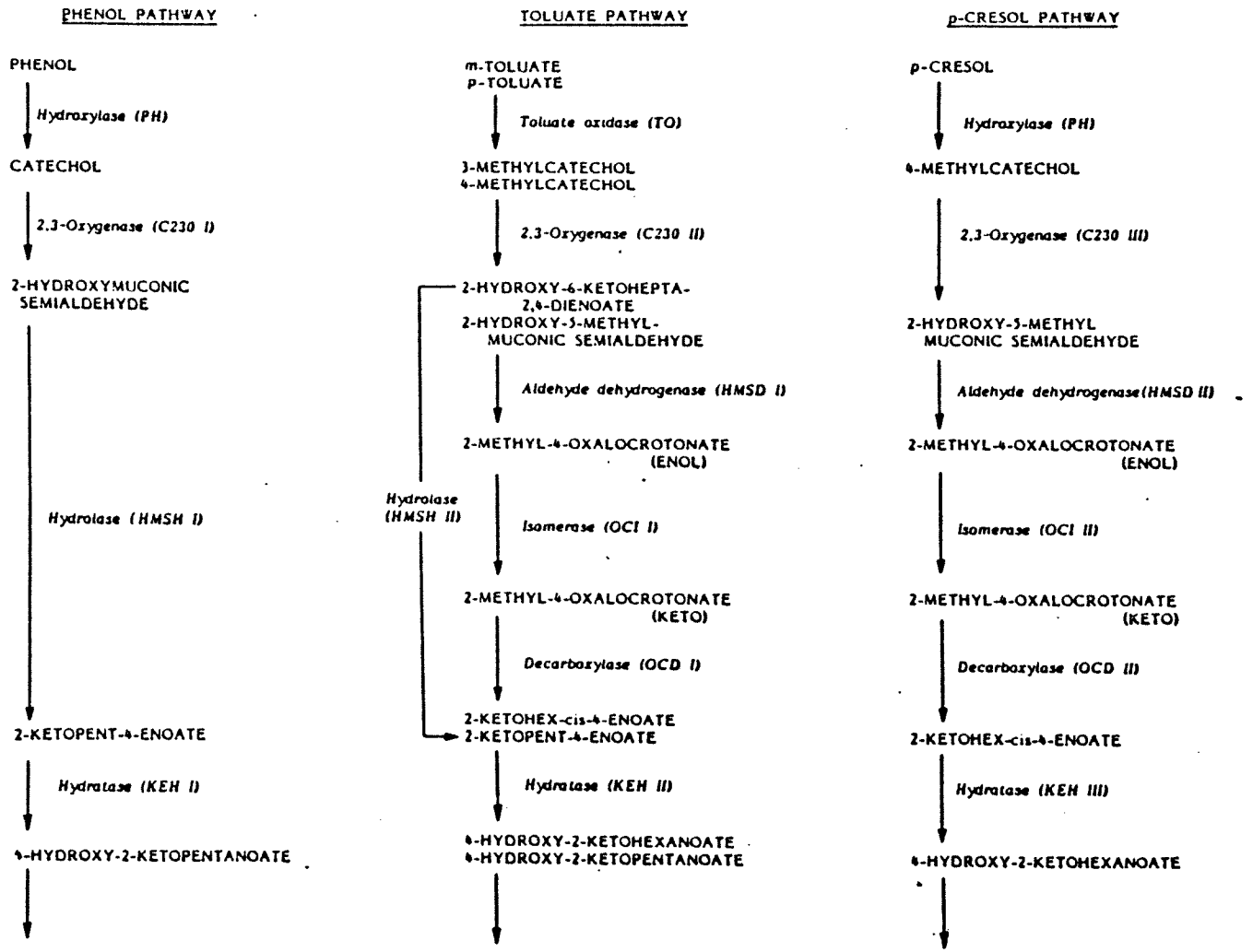
Spontaneous partial revertants of PsU-5 (Bayly, et.al, 1973)

Carbon source used for selection of spontaneous partial revertants	No. of revertants/10^8 cells plated	Growth spectra on replica plating
Phenol	71	9/254 grew on all phenolics; remainder grew only on phenol.
o-Cresol	3[a]	[b]43/44 grew on all phenolics; 1 did not grow on phenol or *p*-cresol.
m-Cresol	1[a]	[b]52/55 grew on all phenolics; 3 did not grow on phenol or *p*-cresol.
p-Cresol	1[a]	[b]35/35 grew on all phenolics.

[a] As only a total of 16 partial revertants were isolated initially on the cresols, differences and similarities within this group are not significant.
[b] Isolated subsequently.

degradation of simple aromatic compounds (benzoate, p-toluate, m-toluate, and phenol). It has been designated as the TOL plasmid. The transfer of this plasmid into organisms which are well characterized biochemically and genetically, allows for the study of the induction pattern of plasmid encoded enzymes (figure 3). A strain of Pseudomonas putida (named PP1-2) was used for their study. It was able to utilize phenol or benzoate as its sole carbon and energy source through the ortho-cleavage pathway. A mutant strain of PP1-2, PP1-3, was unable to grow on either phenol or benzoate as a sole carbon and energy source, and it was lacking in the first enzyme of the ortho-pathway. PP1-3 was able to convert benzoate and phenol to catechol which was subsequently oxidized. The TOL (M1) plasmid was transferred by conjugation from Pseudomonas arvilla mt-2 into PP1-3. Neither strain had the ability to grow on phenol, but PP1-3 with the M1 plasmid was able to grow on phenol (although very slowly). It was found that a mutant of PP1-3 (M1) was able to grow very well using phenol as a sole energy and carbon source. This organism was named PP1-3 (M1-1). The reason for the increased rate of breakdown of phenol was because there was a mutation in the M1 plasmid, and not due to a mutation in the host chromosome. The basis of the mutation was that phenol could then act as an inducer to the plasmid encoded meta-cleavage pathway, but the organism to which the plasmid is transferred must possess an

(figure3)



Pathways for the degradation of phenol, *m*- and *p*-toluate, and *p*-cresol by *A. eutrophus* 345; *p*-cresol is degraded only when the plasmid pRA1000 is cured from strain 345.

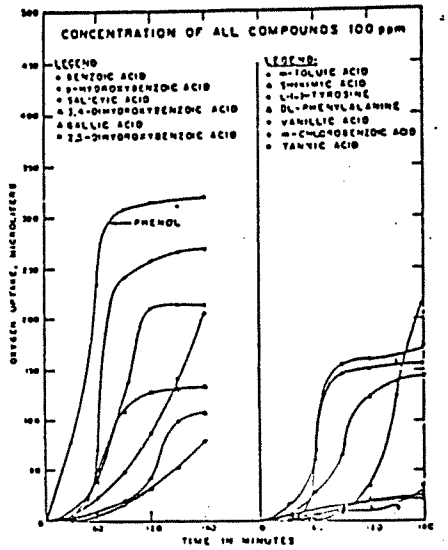
(Hughes, et.al., 1984)

active phenol hydroxylase.

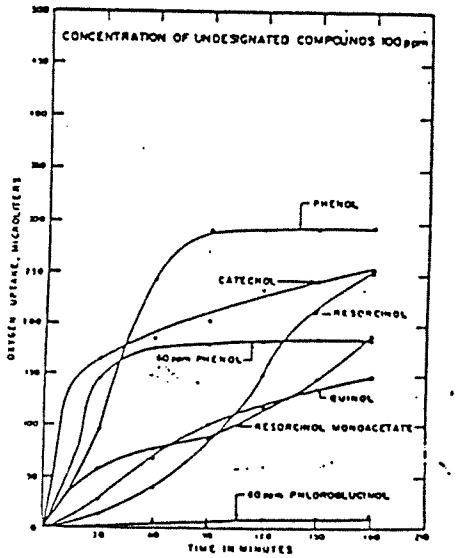
Biochemical studies (Bayly, et. al. [1977]) on wildtype strain PsU-O and its derived mutants suggest that the gene encoding phenol hydroxylase is part of a different operon than that containing the genes for the other enzymes of the meta-pathway. Under all conditions of induction for phenol hydroxylase and catechol 2,3-oxygenase tested, using a strain of PsU-O grown on either fumerate, acetate, pyruvate, or succinate as sole carbon source, the synthesis of the enzymes was non-coordinate. But catechol 2.3-oxygenase and subsequent enzymes appear to be produced coordinately, suggesting that these enzymes of meta-cleavage are located on the same operon while the gene encoding phenol hydroxylase is on a seperate operon. The phenolic compound appears to be the trigger for the formation of all enzymes of meta-fission, because studies on strains defective in phenol hydroxylase (due to structural mutation) induce wildype levels of all of the other enzymes in the pathway after growth on phenol and cresol isomers.

In studying the degradation rate of phenol, by mixed aerobic cultures, and the effect of ring substitutions, Tabak et. al. (1964) used oxygen uptake as a measure of biodegradability (figures 4, 5, 6). The correlation between oxygen uptake and the disappearance of substrate can be seen in Table 6. It was found that phenol-

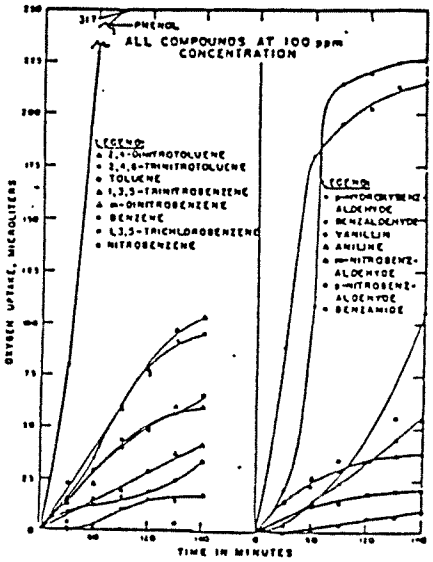
Oxidation of Aromatic Compounds (figure 4)
(Tabak, et.a1., 1964)



Oxidation of benzoic and other acids.

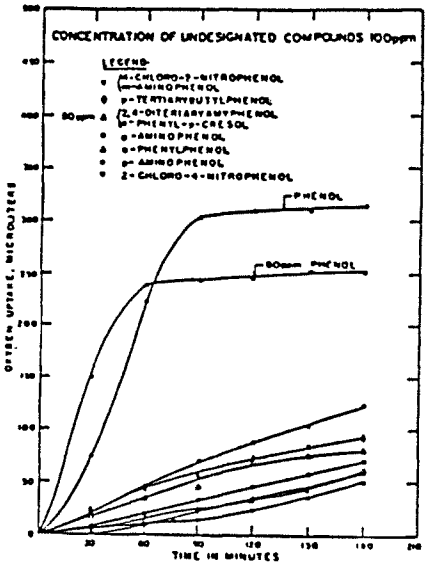


Oxidation of hydroxyphenol derivatives.

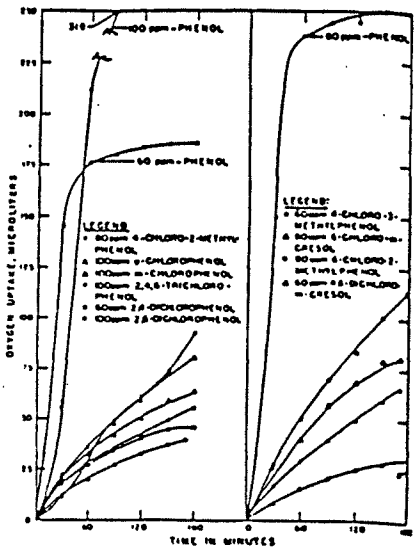


Oxidation of benzenes, anilines, and benzaldehydes.

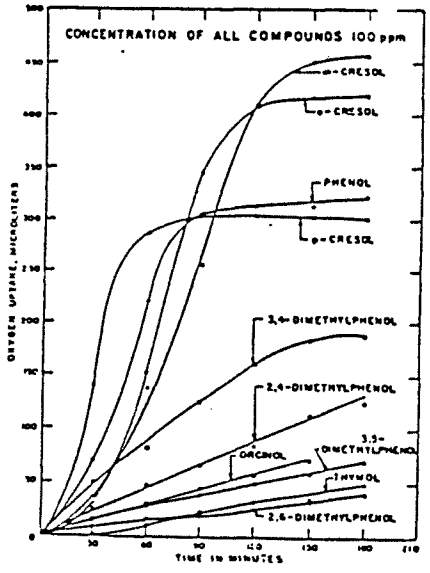
Oxidation of Substituted Aromatic Compounds
(figure 5) (Tabak, et.al., 1964)



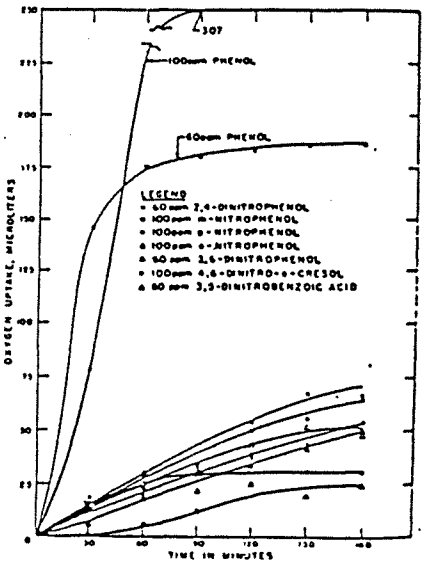
Oxidation of alkyl, aryl, chloro-nitro, and aminophenol derivatives.



Oxidation of chlorophenols and chloro methylphenols.



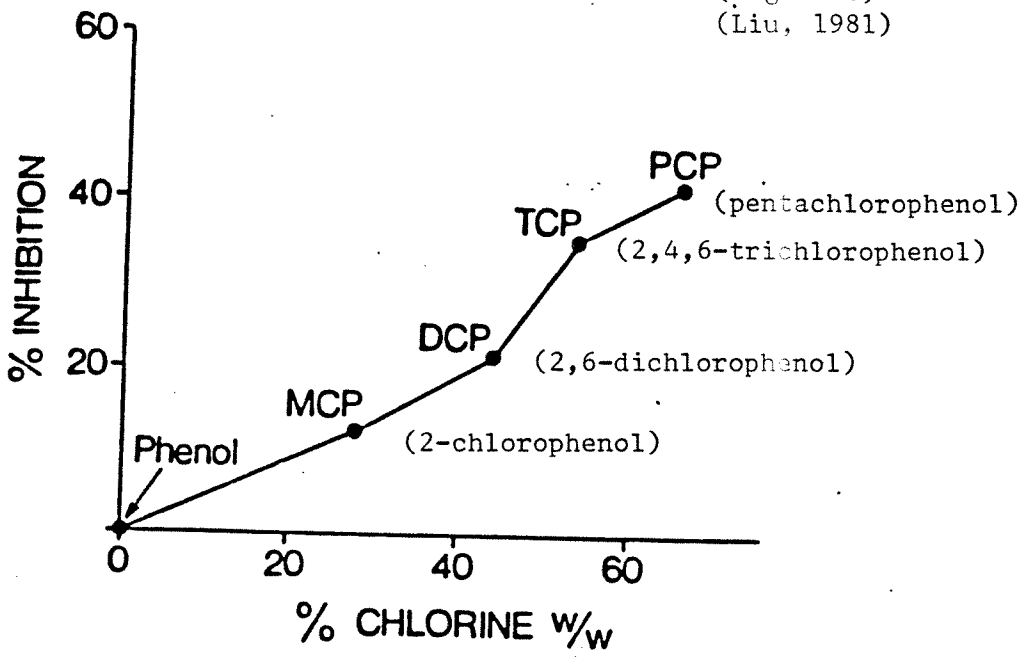
Oxidation of cresols and other methylphenol derivatives.



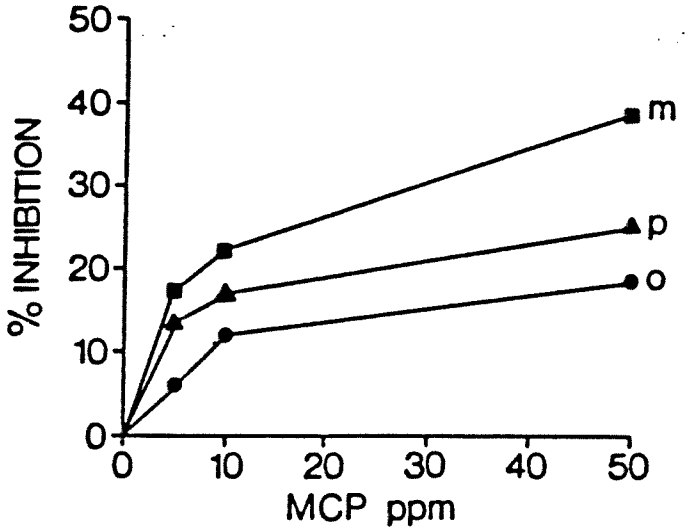
Oxidation of nitro aromatic compounds.

Percent Inhibition of Chlorinated Phenols

(figure 6)
(Liu, 1981)



Percent inhibition as a function of extent of phenol chlorination (see text for abbreviations).



Percent inhibition as a function of monochlorophenols concentration.

adapted cultures had a greater ability to oxidize many of the compounds, but some molecules still remained relatively resistant. Compounds which were resistant to microbial degradation can be seen in Table 7. The differences in resistance appear to be affected by the position of the group on the ring, its size, and its complexity. The presence of a chlorine atom, or nitro group, increased the resistance of phenol and methylphenols. Dichlorphenols were more refractory than monochlorophenols. When the organism came from nutrient broth, there was an observed time delay in growth due to a lag in enzyme induction.

The effect of the degree of chlorination on chemical toxicity to microorganisms can be seen in Table 8 (Liu et. al. (1982)). The toxicities of chlorophenols and other phenolic derivatives correlates to the lipophility (log P) of the compound, which is based upon the octanol/water partition coefficient,P. Toxicity was also a function of the position of the chorine atoms. At 5 ppm, ortho-chlorophenol was half as toxic as para-chlorophenol, and one third as toxic as meta-chlorophenol. These results agree with Kobayashi (1979) who reported that p-CP was about 1.8 times more toxic than o-CP to goldfish. Substitution at both ortho positions (2,6) with chlorine atoms was the least toxic of the dichorosubstitutions, while substitution at both meta positions (3,5) was the most toxic. Liu et al (1982) also developed an empirical equation to predict toxicity:

Table 6

Relationship between O_2 uptake and decomposition of substrate (Tabak, et.al., 1964)

Test compound	Test concn		Amt of O_2 consumed* (endogenous corrected)
	Initial ppm	Last ppm	
			μliters
Phenol	100	99	319
Phenol	80	79	252
Phenol	60	59	186
Catechol	100	97	255
Resorcinol	100	98	252
Quinol	100	86	149
Phloroglucinol	60	3	12
m-Chlorophenol	100	50	66
p-Chlorophenol	100	66	80
2,4-Dichlorophenol	60	18	46
2,6-Dichlorophenol	100	35	39
2,4,6-Trichlorophenol	100	70	56
o-Cresol	100	97	417
m-Cresol	100	97	457
p-Cresol	100	97	306
2,6-Dimethylphenol	100	69	40
3,5-Dimethylphenol	100	37	70
2,4-Dimethylphenol	100	81	126
3,4-Dimethylphenol	100	90	189
Orcinol	100	36	72
Thymol	100	44	48
6-Chloro-*m*-cresol	80	51	81
6-Chloro-2-methylphenol	80	37	66
4-Chloro-2-methylphenol	80	50	90
4-Chloro-3-methylphenol	60	46	113
o-Nitrophenol	100	49	48
m-Nitrophenol	100	39	65
p-Nitrophenol	100	32	54
2,4-Dinitrophenol	60	19	66
2,6-Dinitrophenol	60	8	51
2,4,6-Trinitrophenol	100	28	22
4,6-Dinitro-*o*-cresol	100	60	31
2,4,6-Trinitroresorcinol	60	13	6
2,4,6-Trinitro-*m*-cresol	60	8	11
4-Chloro-2-nitrophenol	100	61	123
2-Chloro-4-nitrophenol	60	7	51
2,6-Dichloro-4-nitrophenol	100	9	35
m-Dinitrobenzene	100	25	12
p-Dinitrobenzene	100	20	32
m-Nitroaniline	100	31	70
2,4-Dinitroaniline	100	39	53
m-Nitrobenzaldehyde	100	27	35
3,5-Dinitrobenzoic acid	100	13	18

* Based on 150 min results with all except orcinol; if endogenous rate was reached sooner,

Table 7

*Compounds resistant to degradation** (Tabak, et.al., 1964)

Phenol derivatives	Benzoic acids	Benzene derivatives
Phloroglucinol	*o*-Nitrobenzoic acid	Chlorobenzene
Pyrogallol	*m*-Nitrobenzoic acid	*o*-Dichlorobenzene
Dimethyldihydroresorcinol	*p*-Nitrobenzoic acid	1,3,5-Trichlorobenzene
2,6-Dichlorophenol	2,5-Dinitrobenzoic acid	Nitrobenzene
2,6-Dinitrothymol	3,4-Dinitrobenzoic acid	*p*-Dinitrobenzene
2,6-Dinitro-*o*-cresol	3,5-Dinitrobenzoic acid	*p*-Nitroaniline
2,4,6-Trinitrophenol	3,5-Dinitrosalicylic acid	*p*-Phenylenediamine
2,4,6-Trinitro-*m*-cresol	2,4,6-Trinitrobenzoic acid	Benzyl alcohols
2,4,6-Trinitroresorcinol	*o*-Chlorobenzoic acid	Benzyl alcohol
4,6-Dichloro-*m*-cresol	*m*-Chlorobenzoic acid	DL-Methylbenzyl alcohol
2,6-Dichloro-4-nitrophenol	*o*-Toluic acid	Miscellaneous compounds
2,4-Dibromophenol	*p*-Toluic acid	Phthalic acid
4-Phenoxyphenol	Syringic acid	DL-Mandelic acid
Benzaldehydes	Vanillic acid	Anthranilic acid
o-Nitrobenzaldehyde	Benzenesulfonic acids	Tannic acid
p-Nitrobenzaldehyde	Benzenesulfonic acid	Benzamide
Benzene	*p*-Toluenesulfonic acid	Cyclohexanol

* Compounds having a maximal endogenous corrected O_2 uptake value below 40 μliters.

Table 8

IC_{50} of various chlorophenols and bromophenols to the culture TL81 expressed in mg L^{-1} and as logarithms of the inverse molar concentrations C[mol L^{-1}] (Liu,et.al., 1982)

Chemicals	IC_{50}(ppm)	$\log \frac{1}{C}$	Chemicals	IC_{50}(ppm)	$\log \frac{1}{C}$
Phenol	2300	-1.39	2,3,4-TCP	13	1.18
2-MCP	700	-0.74	2,3,5-TCP	10	1.30
3-MCP	450	-0.54	2,3,6-TCP	190	0.02
4-MCP	400	-0.49	2,4,5-TCP	12	1.22
2,3-DCP	130	0.10	2,4,6-TCP	240	-0.08
2,4-DCP	75	0.34	3,4,5-TCP	5	1.60
2,5-DCP	85	0.28	2,3,4,5-TTP	4	1.76
2,6-DCP	550	-0.53	2,3,5,6-TTP	54	0.63
3,4-DCP	52	0.50	PCP	9	1.47
3,5-DCP	25	0.81	2-MBP	550	-0.50
			3-MBP	380	-0.34
			4-MBP	400	-0.36
			2,4-DBP	60	0.62
			2,6-DBP	500	-0.30

$$\text{Log } (1/C) = 0.98 \log P - 0.94 \Sigma o - 2.68$$

where: log P = is the lipophility of a compound (P= octanol/water partition coefficient)

Σo = "Hammett's ortho constants" (toxicity decreases with an increase in Hammett's ortho constants)

C = molar concentration (mol L^{-1})

Objective

The study undertaken was the characterization of mixed liquor suspended solids from the Passaic Valley Sewerage Commissioners (PVSC-a 260 MGD facility which receives 55% of its waste from industrial sources), and from the Livingston, NJ municipal treatment plant (a 2.5 MGD facility which receives more than 99% of its waste from domestic sources). Species characterization was to be performed on the raw sludges, and after 10 days exposure to 100 ppm phenol. The PVSC sludge was also to be characterized after 10 days exposure of fresh sludge to 10 ppm 2,6-dichlorophenol; while the Livingston sludge was to be characterized after 10 days exposure to 20 ppm 2-chlorophenol.

Procedures

A. Sludge Acclimation

Tests were performed on four separate batches of PVSC mixed liquor, and two separate batches of Livingston mixed liquor (See Appendix IV). One of the PVSC batches was characterized initially, but not exposed to any phenolics. One of the PVSC batches was characterized microbially after phenol acclimation (but not before). The remaining two PVSC batches were characterized after 2,6-dichlorophenol exposure (without phenol pre-acclimation).

For Livingston sludge, two batches were characterized both before and after phenol acclimation. One of those was then further exposed to 2-chlorophenol and characterized microbially.

Phenol acclimation involved spiking two-liters of mixed liquor to a concentration of approxiamately 100 ppm phenol twice per day on each of ten successive days. Before spiking, the reactor concentration was checked by GC analysis, and in all cases was undetectable (less than 1 ppm). 2,6-DCP exposure involved spiking the reactor with approximately 10 ppm 2,6-DCP over a ten day period. A daily check was made of the residual 2,6-DCP concentration. It was found after the first feed that a residual remained for a period of three days before it disappeared (less than 1

ppm). The reactor was then spiked again, after which it took about 24 hours before no residual remained. From then on the batch was fed twice per day for eight more days. And finally, 2-CP exposure involved spiking the reactor to a concentration of approximately 20 ppm 2-CP also over a ten day period. It was found that the first feed period took 24 hours before no residual (less than 1 ppm) was found by GC analysis. The batch was then spiked twice per day for the next nine days.A batch reactor was used in all cases, and the aeration rate was approximately 1 liter/minute. Details of the GC analysis can be found in Pak (1985).

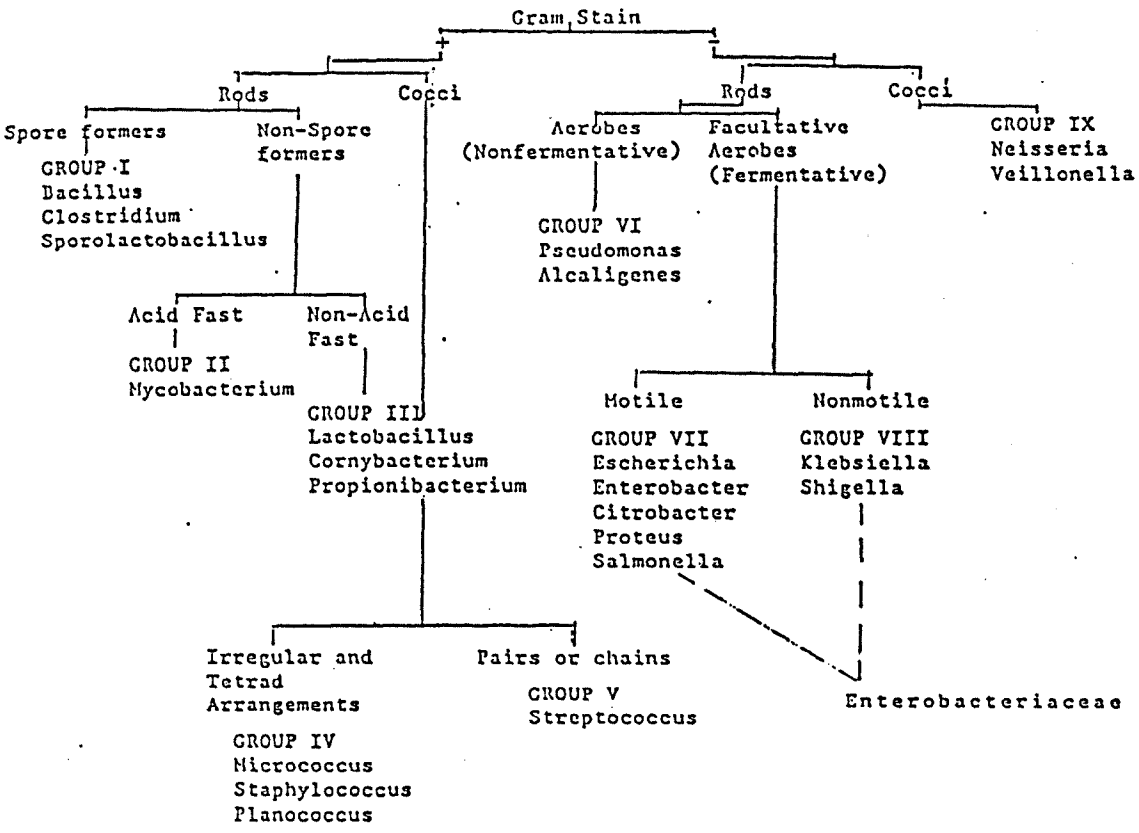
B. Characterization Scheme

A typical scheme for categorizing the different microbial species in a mixed population is given in figure 7. This procedure was modified by the use of commercially prepared diagnostic tubes, as described below. The modified scheme used in the present study is shown in figure 8. For a detail account of the analytical procedure see Boyle (1985).

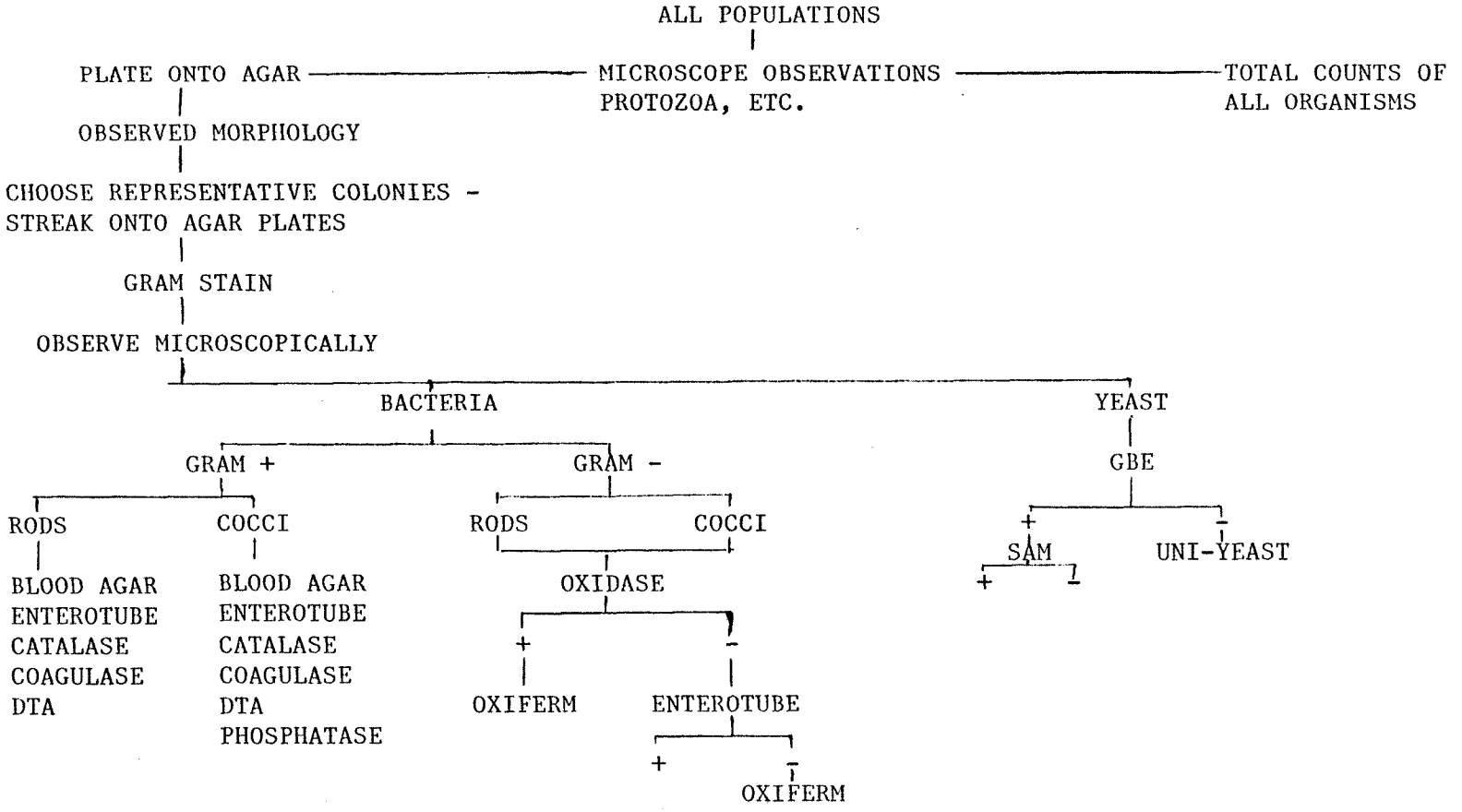
C. Organism Count

A 10 ml sample of mixed liquor was withdrawn from the reactor and transferred into a small, sterilized glass bottle containing 5 grams of five millimeter diameter glass beads of five millimeter diameter. The bottle was capped and

Separation Outline for the Most Common Genera (figure 7)
(Benson, 1979)



Microbial Characterization (figure 8)
(Gneiding, 1984)



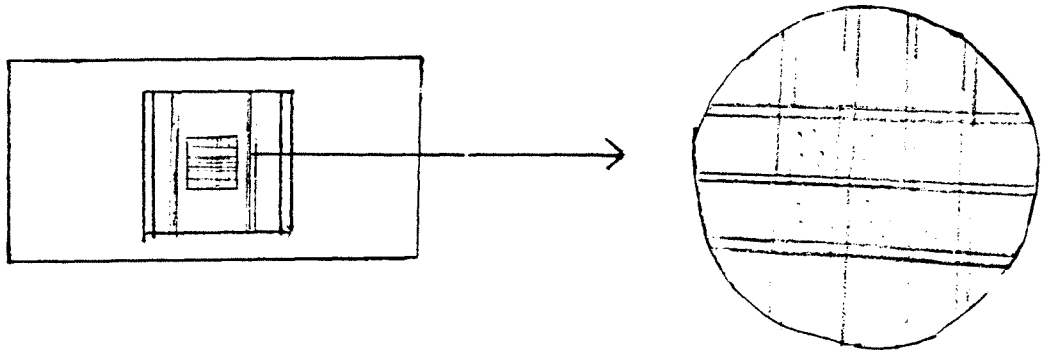
shaken to disperse the organisms. Fifty microliters were then withdrawn and transferred to the center of a "Petroff-Hauser and Helber" counting chamber (figure 9). The counting chamber provides a grid upon which the organisms are counted. Once the dispersed mixed liquor was applied to the chamber, a drop of vaseline was placed on each corner and a coverslip set on top.

The counting chamber was then placed under a microscope (Olympus Phase Contrast #216439) and adjusted until the small grid was brought into focus. The organisms were then counted in twenty grid squares (the volume of a single grid square is 5×10 cm). If the protozoa moved too quickly to count, a drop of formalin was added to the slide to slow them down. The total count of the 20 squares was averaged and multiplied by 2×10 to give the number of organisms per cubic centimeter of solution.

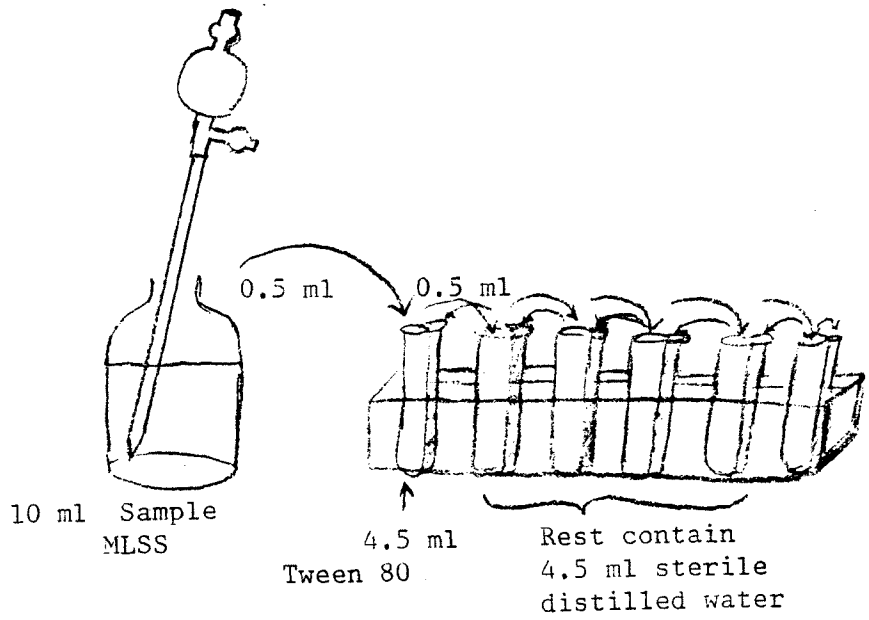
D. Protozoa

From the same dispersed 10 ml sample of mixed liquor that was used for organism count, the protozoa were identified under the microscope. The protozoa were examined and described in as much detail as possible: size, color, method of locomotion, stalked or free swimming. Their sizes were determined by calibrating the slide with a stage micrometer. From this information, and a sketch of each

The Counting Chamber (figure 9)



Dilution Procedure (figure 10)



organism, the protozoa were identified by referring to standard texts (Jahn et al. [1979]; Benson [1979]).

E. Serial Dilutions (see figure 10)

Once again, the dispersed 10 ml sample of mixed liquor was used, this time to perform serial dilutions for microbial plating. Twelve small 10 ml tubes which had been washed and autoclaved (capped with aluminum foil before autoclaving) were set out in a test tube rack. The first tube was filled 4.5 ml of TWEEN 80 (0.1%) solution. Each of the next eleven tubes were filled with 4.5 ml of sterile distilled water (water that had been autoclaved and capped with aluminum foil to prevent contamination). From the 10 ml sample of sludge, 0.5 ml were withdrawn using a sterilized pipet and delivered into the first tube. The tube was shaken, and a sample withdrawn and transferred into the second tube. The second tube was shaken, and 0.5 ml withdrawn and transferred into the third tube. This procedure was repeated throughout the remaining nine tubes. Dilutions produced were 10^{-1}, 10^{-2}, 10^{-3}, . . . 10^{-12}. Dilutions 10^{-1}, 10^{-3}, and 10^{-5} were set aside for yeast and fungal work, while dilutions 10^{-10}, 10^{-11}, 10^{-12} were set aside for bacteria determinations. Bacteria in the original sludge were expected to have a concentration roughly one thousand times greater than yeast and fungi.

F. Bacteria and Yeast Determinations (see Appendices I-III)

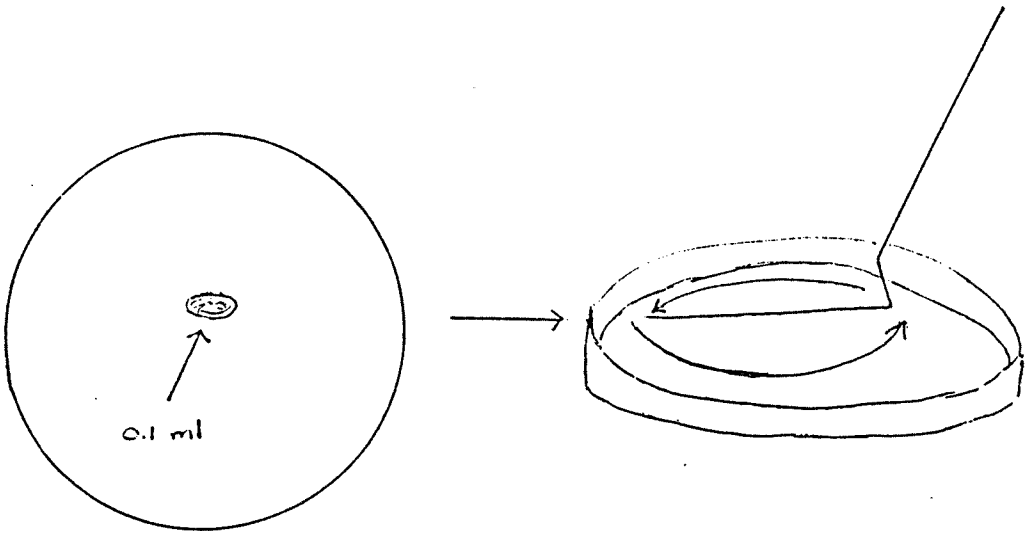
For yeast culture preparations, Czapek with Rose Bengal Oxgall, Sabouraud, Yeast-Malt Extract, and Yeast Nitrogen Base agars which had previously been prepared were labeled with each of the dilutions, 10^{-1}, 10^{-3}, and 10^{-5}. The Plate Count, Floc-Forming, and Cellulose agars were labeled with 10^{-6}, 10^{-9}, and 10^{-12} dilutions for bacteria culture preparations.

To prepare culture plates, a 0.1 ml sample was removed from a specified dilution tube and transferred onto a plate marked with its dilution. For yeast, this resulted in five different agar plates per dilution, and for bacteria three different plates per dilution. The dilution drop on each agar plate was spread using a sterile glass L-rod which was flamed with alcohol (see figure 11).

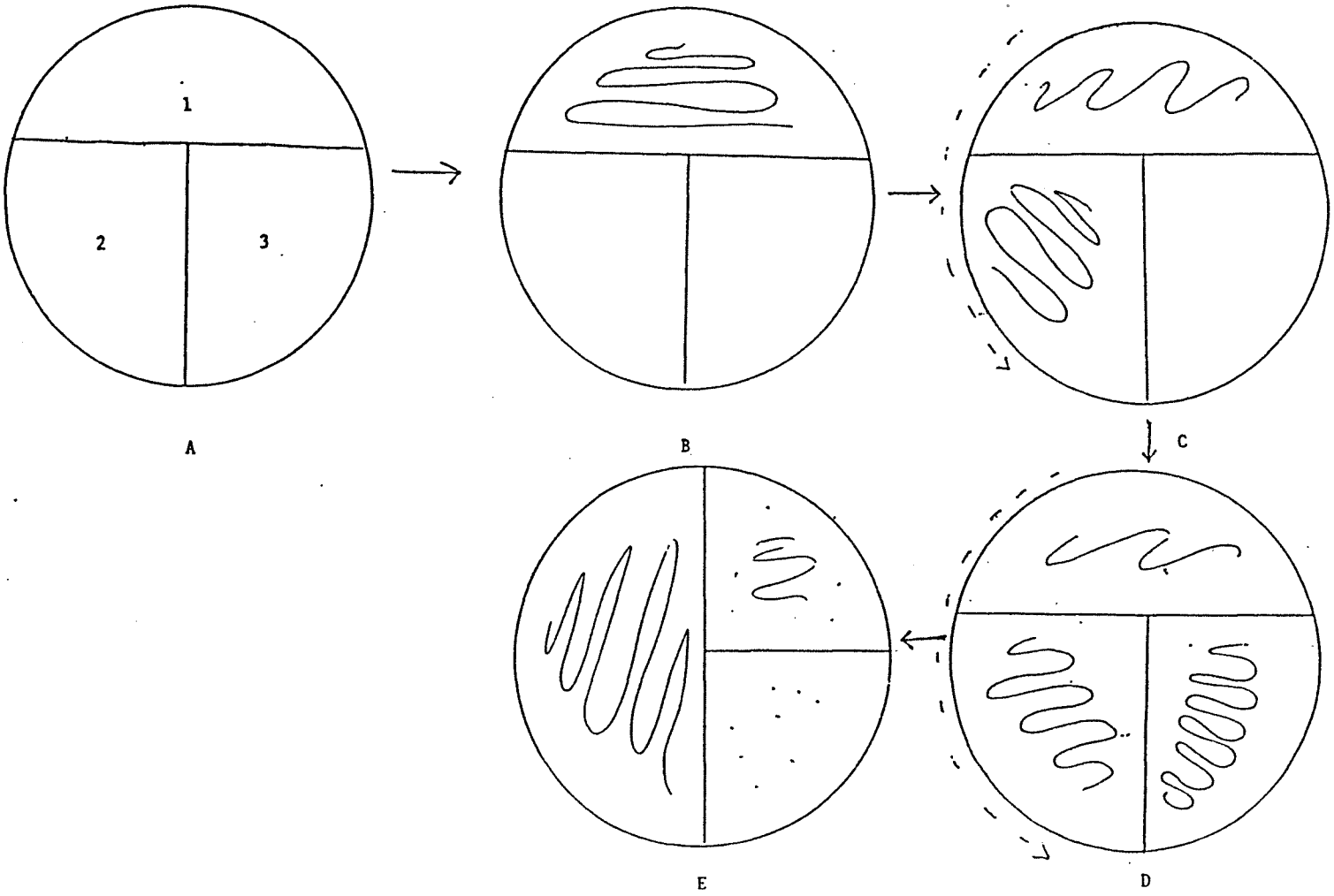
These twenty-four plates were placed into a plastic bag, which was then incubated at room temperature (~26°C) for 48 - 72 hours. After incubation, the bacteria and yeast cultures were examined. Descriptions and numbers of organisms were recorded. The different cultures were then isolated by streaking each one onto a separate plate (see figure 12). The streaked plates were then incubated for 24 hours. This technique was also used for the yeast culture plates.

After the 24 hour-incubation period, the organisms from both bacteria and yeast plates were gram stained. The

Spreading Dilution Drop by Using a Glass L-Shaped Rod
(figure 11)



Procedure for Streaking (figure 12)
(Benson, 1979)



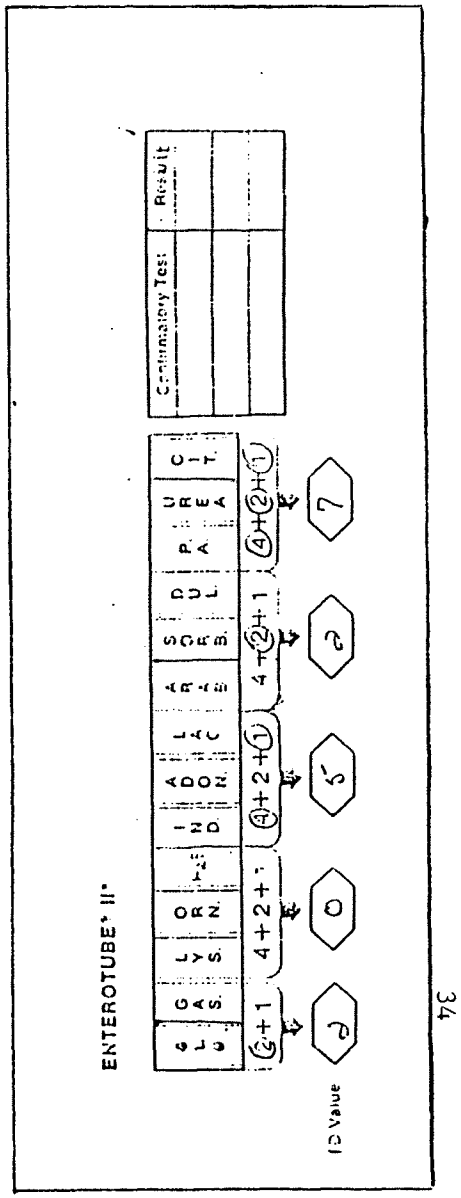
results of gram staining were determined microscopically under oil immersion and recorded. Each of the yeast cultures was also gram stained. This was done to determine their reproductive method, whether by blastospore of pseudohyphae. From this point onward, identification of bacteria and yeast followed separate pathways (see figure 8).

Once bacteria were identified as either gram positive (purple) or gram negative (red), their shapes were classified as either bacilli (rods) or cocci (spheres). Separate identification procedures were required for the gram positive vs. gram negative bacteria. For gram positive bacteria, blood agar was utilized for the growth medium. Classifications were then based on the type of hemolysis produced (see Appendix II). The next step in identification was the inoculation of an Enterotube (figure 13). This tube allows the performance of 15 standard biochemical tests. The test performed are based upon the utilization of certain carbohydrate, nitrogen, and sulfur compounds. A positive result for a test is indicated by a color change, which in turn is a function of pH. This was followed by catalase and coagulase tests, and plating on dextrose-tryptone agar (DTA). These tests are used to characterize the enzyme system produced by the gram positive organisms. The final test is the phosphatase test, performed only upon gram positive cocci. The results of the biochemical tests were

The Enterotube (figure 13)
(Hoffman-LaRoche, 1977)

ENTEROTUBE CODING AND IDENTIFICATION SYSTEM

ID VALUE	ORGANISM	ATYPICAL TEST RESULTS	IDENTIFICATION PROBABILITY	CONFIRMATORY TESTS
20510	ENTEROBACTER AGGLOMERANS	ARA,DUL		
20520	ESCHERICHIA COLI	LYS,ARA	0.6582	SHA -
	SHIGELLA BOYDII	LAC,ARA	0.2536	+
20521	ENTEROBACTER AGGLOMERANS	ARA	0.7864	GLY -
	CITROBACTER AMALONATICUS	ORN,ARA	0.2136	+
20522	ENTEROBACTER AGGLOMERANS	ARA,CIT		
20523	CITROBACTER AMALONATICUS	ORN,ARA	0.6604	GLY +
	ENTEROBACTER AGGLOMERANS	ARA	0.3396	-
20524	ENTEROBACTER AGGLOMERANS	ARA,CIT		
20525	PROVIDENCIA STUARTII	LAC,SOR		
20526	ENTEROBACTER AGGLOMERANS	ARA,CIT		
20527	PROVIDENCIA STUARTII	LAC,SOR	0.8806	XYL -
	ENTEROBACTER AGGLOMERANS	ARA	0.1194	+
20530	ESCHERICHIA COLI	LYS,ARA		
20531	ENTEROBACTER AGGLOMERANS	ARA,DUL		
20533	ENTEROBACTER AGGLOMERANS	ARA,DUL		
20540	ENTEROBACTER AGGLOMERANS	CIT	0.6542	SHA - JTH -
	SHIGELLA BOYDII	LAC	0.2383	+
	ESCHERICHIA COLI	LYS,SOR	0.2075	- +
20541	ENTEROBACTER AGGLOMERANS	NONE	0.7774	ARG -
	ENTEROBACTER SAKAZAKII*	ORN,IND	0.1863	+
20542	ENTEROBACTER AGGLOMERANS	CIT		



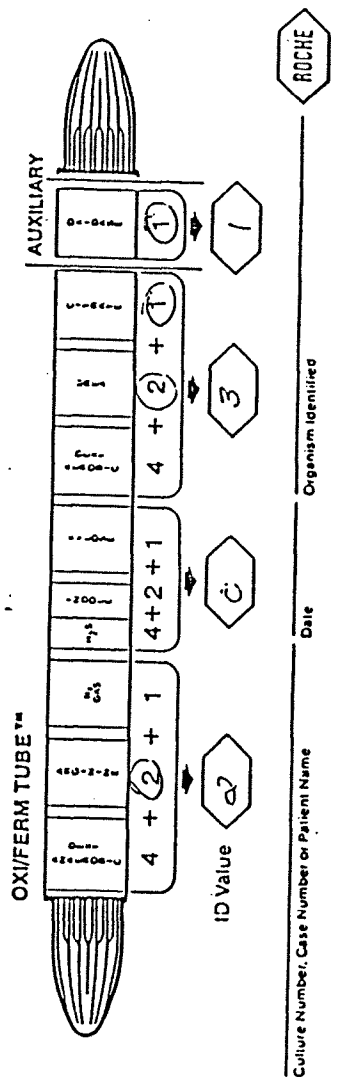
interpreted using Bergey's Manual (7) and the gram positive microoganisms were identified.

The first step in the identification of the gram negative organisms was the performance of the oxidase test for the presence of cytochrome c. Those which were oxidase positive were inoculated into Oxiferm tubes (figure 14). Those which were oxidase negative were inoculated into Enterotubes.

The Oxiferm tube is used for the identification of oxidative fermentative gram negative rods, and allows on a single tube the performance of nine biochemical tests. As with the Enterotubes, a series of substrates is simultaneously inoculated with a culture, and substrate utilization effects pH, which in turn exhibits a color change. These changes are coded for future determination. The Oxiferm tube was incubated at 35-37°C for 18 - 24 hours, and the color changes coded. The same tube was then re-incubated at 35-37°C for another 24 hours, and once again color changes notes for confirmation. The next step was to perform the indole test by adding two to three drops of Kovac's Reagent onto the inside surface of the plastic film of the H_2S/Indole compartment. A positive result was indicated by the development of a red color within a one minute period. The color changes were interpreted using a manual prepared by the manufacturer (15).If more than one

The Oxi/Ferm Tube (figure 14)
(Hoffman-LaRoche, 1977)

ID VALUE	ORGANISM	CONFIRMATORY TESTS		
		WRI	KAN	
1111	ACHROMOBACTER XYLOSOXIDANS	–	–	
	PSEUDOMONAS STUTZERI	V	+	
1121	ACHROMOBACTER SPECIES			
1131	ACHROMOBACTER SPECIES			
		WRI	PB	
1151	PSEUDOMONAS STUTZERI	V	+	
	GROUP 5A-1 Pseudomonas-Like	–	–	
			PB	
1161	ACHROMOBACTER SPECIES BIOTYPE 2		+	
	GROUP 5A-2 Pseudomonas-Like		–	
			PB	
1171	GROUP 5A Pseudomonas-Like		–	
	ACHROMOBACTER SPECIES BIOTYPE 2		+	
		PYO	42	
2011	PSEUDOMONAS FLUORESCENS	–	–	
	PSEUDOMONAS AERUGINOSA	V	+	
2021	ACHROMOBACTER SPECIES BIOTYPE 2			
		PYO	CET	42
2031	PSEUDOMONAS FLUORESCENS	–	V	–
	ACHROMOBACTER SPECIES BIOTYPE 2	–	–	+
	PSEUDOMONAS AERUGINOSA	V	+	+
2040	PSEUDOMONAS MALLEI			



organism was indicated by the code number, confirmatory tests were listed which allowed the determination of the specific species.

If the results of the oxidase test proved negative, an Enterotube was inoculated. This tube was used for the differential identification of gram negative species of the family Enterobacteriaceae. As discussed previously, the tube is inoculated and incubated at 35 - 37° C. for 18 - 24 hours. The positive results, found by comparing an uninoculated tube to an inoculated one, were recorded on the computer coding pad. The results produced a five digit number which was looked up in the computer code book to (14) identify the organism. If more than one organism was identified, confirmatory tests were listed to allow the distinction of the particular species.

For yeast colony identification, once the type of reproduction was found through gram staining, 24 hour isolated streaks were grown on Sabouraud agar. A small sample of the 24 hour colony was inoculated into a GBE tube, which contains a growth broth consisting of beef extract. The inoculated tubes were then incubated for two to four hours at 35 - 37° C. After incubation, a sample was removed and dropped onto a microscope slide. The slide was then examined under low power for the observation of germ tubes (a nonseptated extension from the yeast cell having parallel sides with no constriction). If a germ tube was present, a

SAM tube was inoculated by streaking along the surface of the slant. The SAM tube contains a media composed of sugar and allows the differentiation of the two known organism which have germ tubes. If the SAM tube turned yellow after a four-day incubation at room temp (26° C), the organism was identified as Candida albicans, and if the tube remained purple the organism identified was Candida stellatoidea.

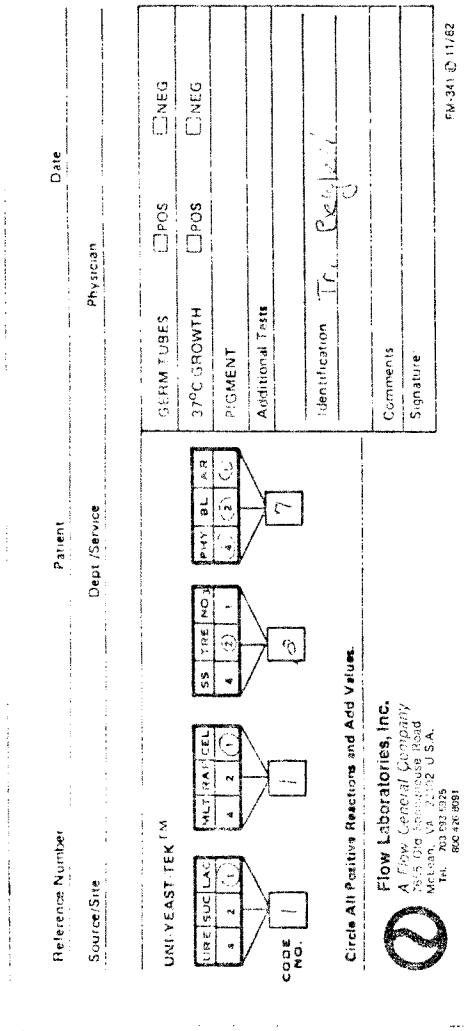
If a germ tube was not found during the microscopic observation, a portion of the 24 hour yeast colony was put into a sterile tube containing 2.0 ml of sterile distilled water and stirred. Using asceptic techniques, 1.5 ml of the suspension was withdrawn and inoculated into the eleven wells of a UNI-Yeast-Tek plate (figure 15). The cornmeal center agar was streaked with the 24 hour yeast colony and a flamed coverslip was set upon the streak. The plate was checked for a seven day period with results recorded upon the reaction pad. Positive results are confirmed by comparison to an uninoculated plate. The organism was identified by the biogram code number using the Uni-Yeast-Tek Computer Code Book supplied by the manufacturer. If more than one organism was listed in the code book for that code number, confirmatory tests were listed to characterize the specific organism.

G. Fungi

Uni-Yeast Tek (figure 15)
(Flow)

CODE	TEST AND RESULTS
1107	URE- SUC- LAC+ MLT- RAF- CEL+ SS- TRE- NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1127	URE- SUC- LAC+ MLT- RAF- CEL+ SS- TRE+ NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1147	URE- SUC- LAC+ MLT- RAF- CEL+ SS+ TRE- NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1167	URE- SUC- LAC+ MLT- RAF- CEL+ SS+ TRE+ NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1307	URE- SUC- LAC+ MLT- RAF+ CEL+ SS- TRE- NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1327	URE- SUC- LAC+ MLT- RAF+ CEL+ SS- TRE+ NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1347	URE- SUC- LAC+ MLT- RAF+ CEL+ SS+ TRE- NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1367	URE- SUC- LAC+ MLT- RAF+ CEL+ SS+ TRE+ NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1507	URE- SUC- LAC+ MLT+ RAF- CEL+ SS- TRE- NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1527	URE- SUC- LAC+ MLT+ RAF- CEL+ SS- TRE+ NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1547	URE- SUC- LAC+ MLT+ RAF- CEL+ SS+ TRE- NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1567	URE- SUC- LAC+ MLT+ RAF- CEL+ SS+ TRE+ NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1707	URE- SUC- LAC+ MLT+ RAF+ CEL+ SS- TRE- NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0
1727	URE- SUC- LAC+ MLT+ RAF+ CEL+ SS- TRE+ NO3- PHY+ BL+ AR+ TR, BEIGLEII 7/1 100.0

1107 - 1727



The next type of organism to be identified were the fungi. This occurred after a five to seven day period of incubation at 26°C. To identify the organism, the edge of the colony was microscopically examined under low power to identify its means of reproduction. The observation was recorded. A sample was then removed, place on a slide, and a few drops of sterile distilled water with lactophenol cotton blue were added which made it easier to distinguish details of the fungal species. From hand-made drawings of the organisms, their method of reproduction, and color of both the surface and underside of the colony, the organism was identified using a standard reference manual (Larone, 1975).

Results & Discussions

Although Livingston is almost entirely domestic and PVSC receives a substantial fraction of industrial wastes, the dominant organisms were basically the same both initially and after exposure to phenolics, (Tables 9 - 12). The general trend with both PVSC and Livingston mixed liquors was toward increasing gram negative dominance (see Tables 9 and 11). The gram positive/gram negative ratio was initially about one to one (0.818 PVSC, and 1.33 Livingston). After feeding with phenol and then the chlorophenols, the ratio shifted toward gram negative species, (0.57 PVSC, and 0.167 Livingston). Furthermore, an initially more diverse population shifted toward the pseudomonads and genera of the family Enterobacteriaceae (including Escherischia, Enterobacter, Citrobacter, Proteus, Salmonella, and Klebsiella). The general characteristics of these organisms are: gram negative rods, facultative aerobes, and fermentative.

Upon addition of 2,6-dichlorophenol to the PVSC mixed liquor, there was a pH shift from 7-8 to 6-7 (determined by using litmus strips) which enhanced the environment for yeasts and fungi relative to bacteria.

Through microscopic observation, the Livingston yeast population also appeared to increase in number following the

Table 9

Predominant Genera in Livingston Mixed Liquor

FRESH	PHENOL	2-CHLOROPHENOL
1.8×10^{10} organisms/ml	1.6×10^{10}	3.8×10^{10}
gram +/gram - = 1.33	0.55	0.167
Bacillus →		
Micrococcus →		
Staphylococcus →		
Achromobacter →		
Acinetobacter →		
Alcaligenes →		
Citrobacter →		
E. coli →		
Enterobacter →		
Group M-4F Moraxella-like →		
Group 5E-1 Pseudomonas-like		
Proteus →		
Providencia →		
Pseudomonas →		
Serratia →		
Xanthomonas →		
Actinomyces →		
Streptomyces →		
Saccharomyces		
Aspergillus		
Gliocladium →		
Microsporium		
Mucor →		
Penicillium →		
Rhizopus		
Syncephalostrum		
Trichophyton		
Vorticella →		
Pseudopodia		
Stalked species →		
Flagellates →		
Ciliates →		
Invertebrates		
Algae		
Rotifers →		

Livingston Results of Yeast Species (Table 10)

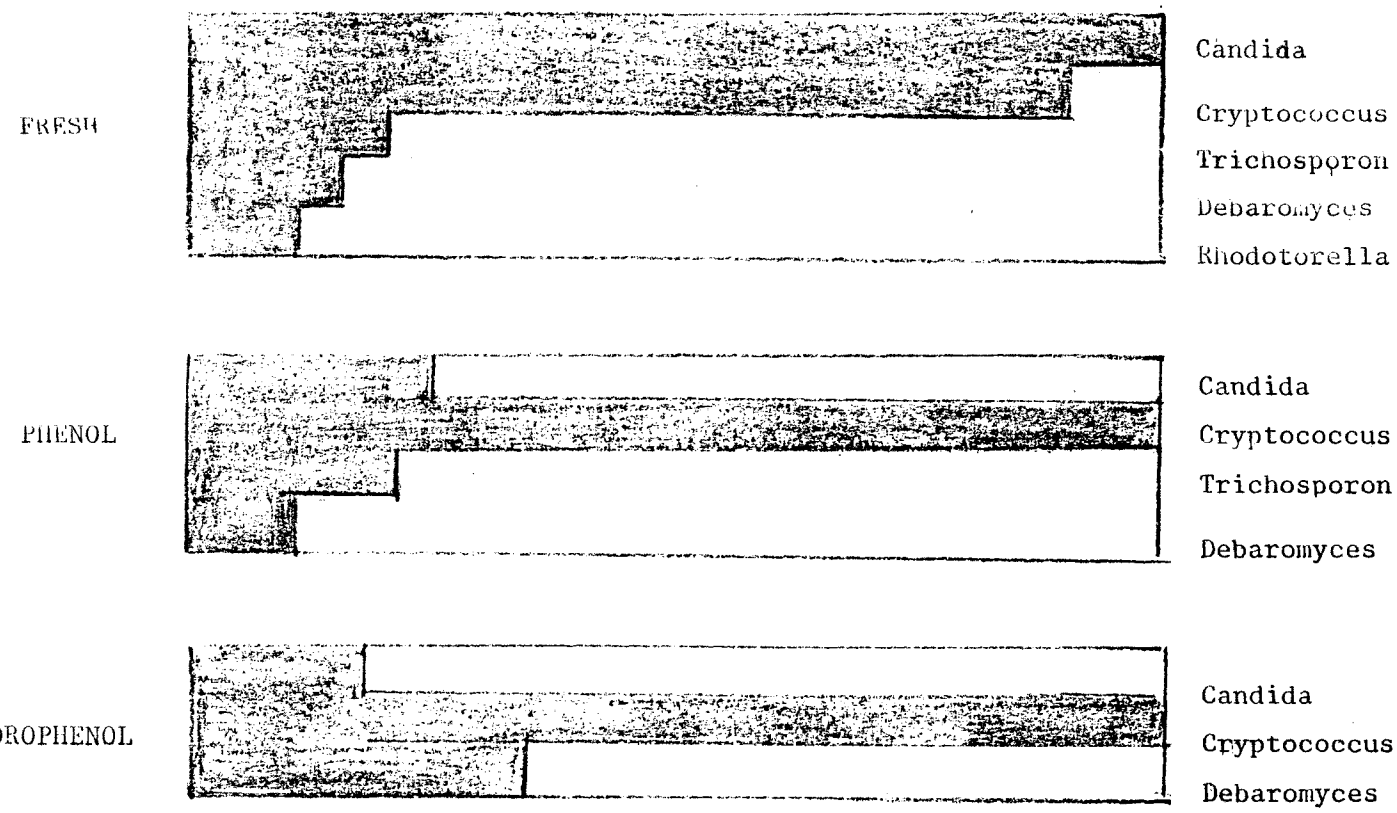
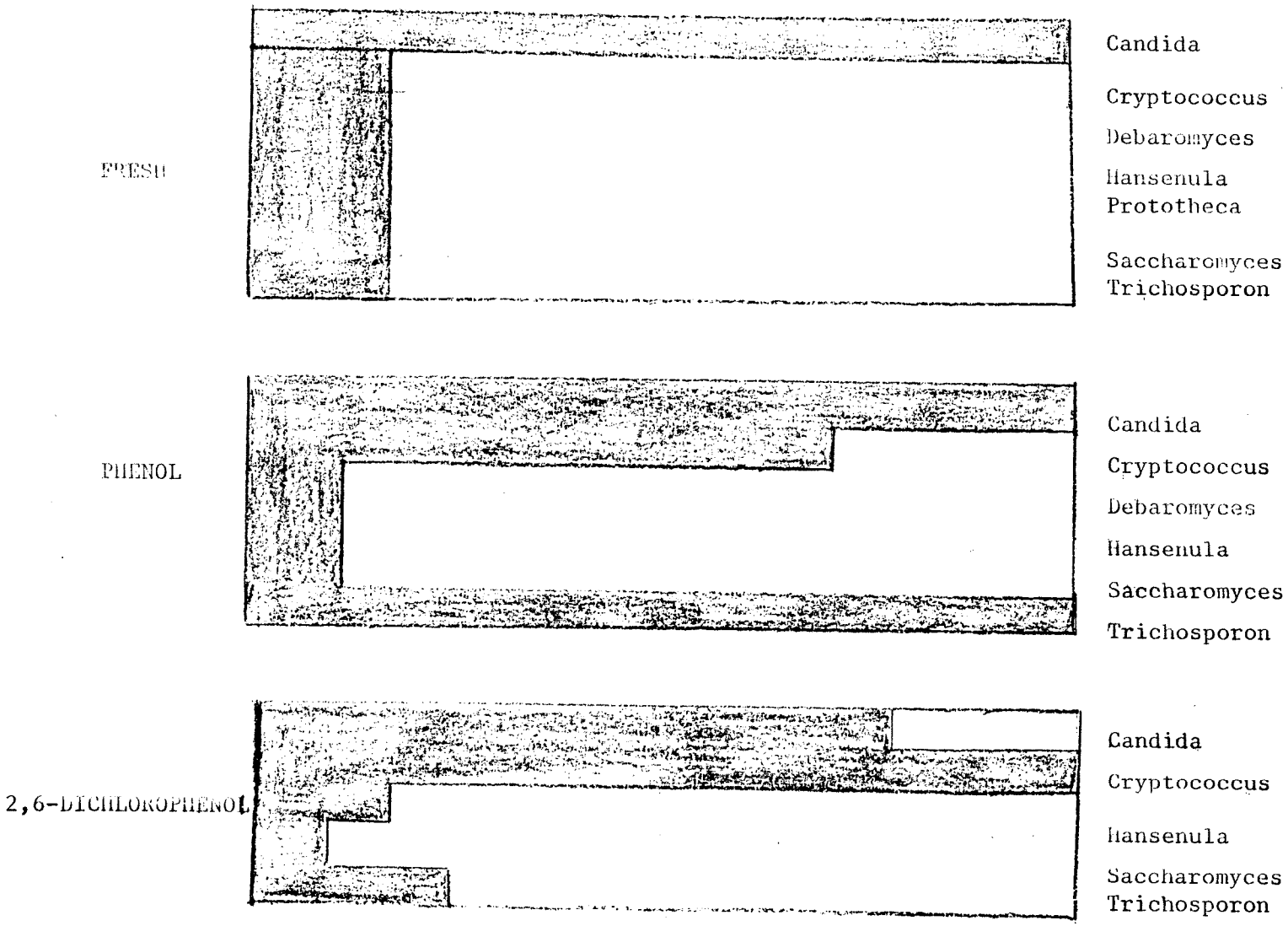


Table 11

Predominant Genera in PVSC Mixed Liquor

FRESH	PHENOL	2,6-DICHLOROPHENOL
2.34×10^{10} organisms/ml	2.2×10^{10}	2.1×10^{10}
gram +/gram - = 1.33	0.75	0.57
Bacillus →	→	→
Micrococcus →	→	→
Staphylococcus →	→	→
Achromobacter →	→	→
Acinetobacter →	→	→
Citrobacter		
Enterobacter →	→	→
Pasteurella →	→	→
Pseudomonas →	→	→
Shigella		
E. coli →	→	
Moraxella →	→	
Group M-4 Moraxella-like →	→	→
Group 2K-1 Pseudomonas-like →	→	→
Serratia →	→	→
Alcaligenes →	→	→
Providencia →	→	→
Actinomyces		
Aspergillus →	→	
Chaetonium		
Cladosporum →	→	
Epidermophyton →	→	
Gliocladium →	→	→
Helminthosporon		
Microsporum →	→	
Monilla		
Penicillium →	→	→
Scopulariopsis		
Streptomyces →	→	→
Trichophyton →	→	
Monosporium		
Curvularia		
Fonsecaea		
Mucor		
Rhizopus		
Geotrichum →	→	→
Sepedonium		
Trichoderma		
Pseudopodia →	→	→
Stalked species →	→	→
Flagellates →	→	→
Ciliates →	→	
Algae		
Rotifers		

Passaic Valley Sewage Commission Results of Yeast Species (Table 12)



feeding with 2-chlorophenol, which may have been partly due to the slight decrease in pH.

The protozoa also go through a shift in the dominant species present. In PVSC, the emphasis was on the stalked organisms, the majority being Vorticella, but also including a variety of other types (see Table 11). After treating with 2,6-dichlorophenol, free swimmers (such as Chlamydomonas [which constituted the majority of such types], Euglena, Pandorina, and Trichamoeba) comprise a large percentage of the total protozoa population.

In the Livingston mixed liquor, there were a variety of pseudopodia, ciliated, flagellated, and stalked organisms (Ceremonas, Euglena, Paramecium, Volvox, Carchesium, Zoothamnium, and the majority being Vorticella) and even nematodes. After feeding with phenol, there was a slight shift toward ciliated and flagellated protozoa. After feeding with 2-chlorophenol, primarily ciliated and flagellated free swimmers remained (Table 9).

In all cases, the total organism count also decreased after feeding with the phenolic compounds. Bacterial deaths may have contributed temporarily to the apparent increase in the protozoan population. A more likely reason may be that stalked organisms could have broken apart, with the resulting free-swimmers contributing to an apparent increase in protozoa. A detailed list of species is given in Appendix IV.

In comparing the results of the present work to those obtained previously with the Livingston sludge (Gneiding, 1984), Pasteurella, and Ceremonas were not found in the present work (although they had been noted previously), while Group 4-M Moraxella-like and Group 5E-1 Pseudomonas-like, were identified in the present work (but were not found in the prevous study). It was also found that the ratio of gram +/gram - organisms varied. While the fresh batch showed an approximate ratio of one to one the results after treating with phenol or chlorinated phenols showed a greater percentage of gram negative organisms which is different than that found by Gneiding (1984), but is the same trend that was discovered by Boyle (1985).

In comparing the results obtained in the present work on the PVSC sludge with those of Boyle (1985) Alcaligenes and Rhodotorula were not found in the present work (although they had abeen found in the previous study,) while Chaetonium, Cladosporum, and Epidermophyton were found in the present study (but not by Boyle). It is difficult to compare the organism count because only a total organism count was performed and not a count on each type of organism so the general trend of bacteria, yeast, and protozoa cannot be seen.

These discrepancies my be due to the variability between batches, or the variability between investigators utilizing fairly complex and somewhat subjective tests.

Conclusion

The microbial characterization of Passaic Valley and Livingston mixed liquor was performed on fresh sludge, after feeding with phenol (100 ppm for 10 days), and again after feeding with either 2-chlorophenol (20 ppm for 10 days) or 2,6-dichlorophenol (10 ppm for 10 days). The identification of the yeast, bacteria, fungi, and protozoa present in the sludge was based upon physiological and biochemical characteristics. It was found that there was a bacterial population shift toward pseudomonads and members of the family Enterobacteriaceae, while the yeast shift was toward Cryptococcus and Candida species. The protozoan population shift from primarily stalked organisms toward free swimmers.

In spite of the significant differences in operation of the two treatment plants, results were very similar for the mixed liquor populations.

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Appendix I

Formulae for Plating Media

Cellulose Decomposing Bacteria Media:

$(NH_4)_2SO_4$	0.1 gram
K_2HPO_4	0.1 gram
$MgSO_4 \cdot H_2O$	0.05 gram
$CaCO_3$	0.2 gram
NaCl	trace
Agar	2.0 grams
Distilled Water to	100 ml

Autoclave. When cool, pour into plates and add a strip of filter paper into each one.

Floc-Forming Bacteria Medium:

Protease-peptone	0.2 gram
Yeast Extract	0.1 gram
Agar	2.0 grams
Distilled Water to	100 ml

Plate Count Agar:

Bacto-Tryptone	5 grams
Yeast Extract	2.5 grams
Dextrose (Glucose)	1 gram
Agar	15 grams
Distilled Water	1000 ml

or

Difco Plate Count Agar	23.5 grams
Distilled Water	1000 ml

Czapek (or Cornmeal) Agar with Rose Bengal:

Saccharose	30 grams
Na_2NO_3	2 grams
Dipotassium Phosphate	1 gram
Magnesium Sulfate	0.5 gram
Potassium Chloride	0.5 gram
Ferrous Sulfate	0.01 gram
Agar	15 grams
Distilled Water	1000 ml

equivalently

Czapek Powder	4.9 grams
Distilled Water	100 ml

Autoclave. When cool add 33 mg/100 ml solution of Rose Bengal. Mix, then pour into Plates.

Oxgall Medium:

Peptone	10 grams
Glucose	10 grams
Oxgall (bovine bile)	15 grams
Agar	20 grams
Distilled Water to	1000 ml

Add 50 mg of streptomycin and 10 mg penicillium G after autoclaving. Filter Sterilize antibiotics.

Sabouraud Agar:

Peptone	1.0 gram
Glucose	4.0 grams
Agar	1.5 grams
Distilled Water to	100 ml

Yeast-Malt Extract Agar:

Yeast Extract	0.3 gram
Malt Extract	0.3 gram
Peptone	0.5 gram
Glucose	1.0 gram
Agar	2.0 grams
Distilled Water to	100 ml

After autoclaving, cool to approximately 50^{o}C and add 0.7 ml of 1 N HCl.

Yeast Nitrogen Base Agar:

Bacto Yeast Nitrogen Base	6.7 grams
Dextrose/Sucrose	5 grams
Agar	1.5 grams

Distilled Water	100 ml

All agars are heated to boiling to get agar into solution. Then autoclaved at 121°C for 15 minutes.

Appendix II

Specific Test Procedures

Gram Stain:

A. Hucker's(Modified) Crystal Violet:

SOLUTION A:

Crystal Violet (Certified)	2 grams
Ethyl Alcohol, 95%	100 ml

SOLUTION B:

Ammonium Oxalate	0.8 gram
Distilled water	80.0 ml

Mix solutions A and B. Store for 24 hours before use. Filter through paper into staining bottle.

B. Counterstain:

STOCK SOLUTION:

Safranine O (certified)	2.5 grams
Ethyl Alcohol, 95%	100 ml

WORKING SOLUTION:

Stock solution	10 ml
Distilled Water	90 ml

C. Decolorizing Agent:

Technical Acetone	100 ml
Isopropyl Alcohol	400 ml

Take a sample of 18 - 24 hour old colony. Place on slide with a drop of sterile distilled water. Let air dry. Then heat fix. Flood slide with Crystal Violet and let stand for twenty seconds. Rinse with distilled water. Flood slide with Gram's Iodine for one minute. Then rinse with decolorizing agent until flows colorlessly, about 10 - 20 seconds. Wash with distilled water. Next flood the slide with Safranin stain. Let sit for twenty seconds. Rinse with distilled water. Allow to air dry then examine under oil emersion.

Oxidase Test:

The oxidase test reagent is placed on a piece of

Whatman #1 filter paper in a petri dish. Then a sample of an isolated 18 - 24 hour old bacteria colony is placed on the filter paper. The whole clump of bacteria will turn purple in a positive test. The reagent is prepared by adding 0.2 grams dimethyl-p-phenylenediamine hydrochloride in twenty ml of distilled water. The test works because the organism contains an oxidase enzyme which in the presence of atmospheric oxygen, cytochrome c, and an oxidase reagent, oxidize the reagent to form a colored compound. The test actually seeks the presence of cytochrome c and is positive only for those bacteria containing respiratory compound, cytochrome c (MacFaddin 1980).

Catalase Test:

A sample of the 18 - 24 hour culture is placed on a piece of filter paper in a petri dish. A drop of 3% hydrogen peroxide is dropped upon it. If it bubbles right away, the test is positive. The test works because the hydrogen peroxide is converted into water and oxygen gas by the catalse enzyme found in most cytochrome containing aerobic and facultative bacteria (MacFaddin 1980).

Phosphatase Test (Gram ± Cocci):

One ml of 1% Seitz filtered phenolphthalein phosphate is added to 100 ml of Nutrient Agar and poured into plates.

Innoculate plate with 18 - 24 hour old isolated bacteria colony and incubate for 24 hours at 37 C. Then expose the plates to ammonia vapors. Colonies of phosphatase positive Staphylococci willturn pink due to free phenolphthalein. Staphylococcus aureus is positive. Coagulase negative Staphylococcus and Micrococcus are usually phosphatase negative. To make the 1% solution, add one gram of phenolphthalein diphosphate sodium salt to 100 ml of distilled water. Sterilize by Seitz filtration.

Dextrose Tryptone Agar:

Tryptone	1.0 gram
Dextrose	0.5 gram
Bromeresol Purple	0.0004 gram
Agar	1.5 grams
Distilled Water to	100 ml

Coagulase Test:

A small sample of an isolated bacteria colony is well mixed with sterile distilled water on a clean glass slide. The slide is observed for self-agglutination. If this does not occur, a small amount of lypholized rabbit plasma is added to the bacterial suspension. If clumping occurs, the presence of coagulase enzyme is present; this results in a positive test.

Indole Test:

Kovac's Reagent:	Para-dimethyl-amino-benzaldehyde	5.0 gms
	Alcohol, amyl or butyl	75.0 gms
	Hydrochloric acid, concentrated	25.0 gms

Indole is produced upon the metabolism of typtophan by the enzyme tryptophanase. A positive result will be upon the formation of a pink color upon the addition of Kovac's Reagent. The reaction occurs because indole, if present, will combine with the aldehyde to give a red color in the alcohol. This reaction takes place because the acid splits the protein.

Trypticase Soy Agar:

Bacto-Tryptone	15 grams
Bacto-Soytone	5 grams
NaCl	5 grams
Agar	15 grams
Distilled Water	1000 ml
or	
Trypticase soy agar powder	40 grams
Distilled Water to	1000 ml

Appendix III

Confirmatory Tests

Cetrimide Agar:

Gelysate Pancreatic Digest of Gelatin	2.0 grams
$MgCl_2$	0.14 gram
K_2SO_4	1.0 gram
Cetrimide	0.03 gram
Distilled Water	100 ml

Leifson Flagellar Stain:

Pararosaniline Acetate	0.9 gram
Pararosaniline Hydrochloride	0.3 gram
95% Ethyl Alcohol	100 ml
Tannic Acid	3.0 grams
Distilled Water	100 ml
NaCl	1.5 grams
Distilled Water	100 ml

Prepare stain by making three solutions:

1. 1.5% NaCl in distilled water
2. 3.0% Tannic Acid in distilled water
3. 1.2% Dye mixture. Allow dye mixture to stand overnight at room temperature to completely dissolve.

Mix equal volumes of the three solutions, shake, and let stand for 2 hours. Store in a tightly stoppered bottle in the refrigerator. Precipitate which settles upon storage should not be disturbed. Solution can be stored up to two months in refrigerator and indefinately in freezer. Frozen stain should be throughly mixed after thawing; after mixing allow precipitate to settle.

Cultures are grown in BHI broth or suitable peptone broth at room temperature for 18 - 24 hours. Formalin (0.25 ml) is added to 4 ml of overnight broth and allowed to stand for 15 minutes after mixing. Tube is filled with fresh distilled water, mixed, and centrifuged. Remove supernatant carefully be decanting. Add distilled water, mix, and recentrifuge. Remove supernatant, resuspend organisms in 1 - 2 ml of distilled water, and dilute until suspension is barely turbid.

Heat a clean slide and mark 1/3 and then 2/3 from one end using a grease pencil. Place a large loopful of bacterial suspension on slide, then tilt and allow suspension to flow. Allow the suspension to air dry. DO NOT HEAT FIX.

One milliliter of supernatant stain which is warmed to room temperature, is applied to slide. As alcohol evaporates, a precipitate

forms in the solution, 5-15 minutes. As soon as precipitate forms over the entire smear, staining is completed and stain is carefully washed off slide by flooding with water. Air dry.

Blood Agar:

Blood Agar Base (Difco)	40 grams
Distilled Water	1000 ml
or	
Heart Muscle, infusion from (solids)	10 grams
Peptic Digest of Animal Tissue	10 grams
NaCl	5 grams
Agar	15 grams
Distilled Water	1000 ml

After autoclving, cool to 45°C and add 5% sterile, defibrinated blood, mix well and pour into plates.

TO DEFIBRINATE BLOOD:

Draw whole blood aseptically with syringe equipped with 18-gauge needle (to prevent hemolysis by mechanical damage) and disperse blood immediately into sterile flask containing a layer of sterile glass beads (3 mm). Shake flask side to side for 10 minutes. Fibrin formed during clotting will be deposited on the beads. Decant supernatant containing blood cells and serum into sterile container and store in refrigerator.

This test is performed to determine the hemolytic activity of those bacteria which are difficult to grow, Staphylocccus, Streptococcus, and Diplococcus. The activity is noted by a green zone around the colony, alpha hemolysis, a clear zone, beta hemolysis, or no hemolysis known as gamma hemolysis.

Lactophenol Cotton Blue:

Lactic Acid	20 ml
Phenol Crystals	20 grams
Glycerol	40 ml
Distilled Water	20 ml
Cotton Blue (poirrier's Blue)	0.05 gram

dissolve phenol in the lactic acid, glycerol, and water by gently heating. Then add cotton blue. This solution is used as mounting fluid and stain. The lactic acid preserves the fungal spores, the phenol is a killing agent, and the cotton blue

Brain Heart Infusion Agar:

Calf Brains, infusion from	15.76 grams
Beef Heart, infusion from	19.70 grams
Protease Peptone, Difco	0.79 gram
Dextrose	0.158 gram
NaCl	0.39 gram
Disodium Phosphate	0.20 gram
Distilled Water	1000 ml

MacConkey Agar:

Peptone	17 grams
Protease Peptone	3 grams
Lactose	10 grams
Bacto-Bile salts No.3	1.5 grams
NaCl	5 grams
Agar	13.5 grams
Bacto-Neutral Red	0.03 gram
Bacto-Crystal Violet	0.001 gram
Distilled Water	1000 ml

or

MacConkey Agar Powder	10 grams
Distilled Water	1000 ml

Motility Test Medium:

Beef Extract	3 grams
Peptone	10 grams
NaCl	5 grams
Agar	4 grams
Distilled Water	1000 ml

Nutrient Broth:

Peptone	5.0 grams
Beef Extract	3.0 grams
Distilled Water	1000 ml

pH 6.8 $\pm$ 1

Nutrient Agar:

Make Nutrient Broth and add 15 grams of Agar per 1000 ml.

Nutrient Gelatin:

Make Nutrient Broth and add 12% gelatin.

Appendix IV

Livingston: Fresh (6/27/85)	(9/13/85)
Total: 1.798×10^{10} organisms/ml	Total: 1.42×10^{10} organsims/ml
+/- : 1.43	+/-: 1.23
FUNGI:	FUNGI:
Actinomyces	Alternia sp.
Aspergillus	Microsporum nanum
Gliocladium	Penicillium
Mucor	Streptomyces
Penicillium	Verticillum
Rhizopus	
Saccharomyces cerevisiae	
Syncephalostrum	
Trichophyton schoenleini	
? Trichophyton terrestre	
YEAST:	YEAST:
Candida albicans	Cryptococcus albidus var albi
Candida famata	Cryptococcus laurentii
Candida intermedia	Debaromyces hansenii
Cryptococcus albidus var albi	Trichosporon pullulans
Cryptococcus gastricus	
Cryptococcus laurentii	
(Debaromyces hansenii)	
Rhodotorula pilimanae	
Trichosporon pilimanae	
Trichosporon beigleii	
Trichosporon pullulans	
BACTERIA:	
Acinetobacter anitratus	Acinetobacter lwoffii
Acinetobacter lwoffii	Alcaligenes faecalis
Enterobacter agglomerans	Citrobacter amalonaticus
Enterobacter cloacae	Citrobacter freundii
Enterobacter sakazakii	Enterobacter agglomerans
E. coli	Enterobacter cloacae
Group 5E-1 Pseudomonas-like	Proteus vulgaris
Group M-4F Moraxella-like	Pseudomonas alcaligenes
Pseudomonas cepacia	Pseudomonas flourescens
Pseudomonas flourescens	Pseudomonas maltophilia
Pseudomonas putida	Pseudomonas putida
Serratia liquefaciens	Serratia liquefaciens
Bacillus	Bacillus
Micrococcus	Micrococcus
Staphylococcus	Staphylococcus aureus
Staphyloccus aureus	

LIVINGSTON: Fresh (continued)

PROTOZOA:
Ceremonas (amoeboid, 2 flagella)
Chrysococcus (flagellate)
Dinobyron (2 unequal flagella)
Eudorina (spherical colony;1,2,4 flag.)
Euglena (free swimmer w/ locomotor flag.)
Peridinium (Dinoflag. w/ 2 flagella)
Synura (2 flag.,motile)
Tetraspora (algae)
Trichoamoeba (pseudopodia & filaments)
Volvox (spherical, external flag.)
Vorticella (stalk)
Zoothamnium (like vorticella, but branching colonies; contracts as unit ciliated and stalked)

PROTOZOA:
Baileyia mutabilis (pseudopodia)
Carchesium polypinum(stalk & cilia)
Euglena (free swimmer, flagella)
Heteromena acus (2 flag.)
Nematodes (microscopic invert.)
Paramecium (ciliated, free swim.)
Petalmonomadidae (1 flag.)
Polychaeos dubium (pseudopodia)
Synure (2 flag, motile)
Volvox (spherical, external flag.)
*Vorticella (stalked and ciliated)

LIVINGSTON: PHENOL ACCLIMATED (7/29/85)	(9/25/85)
Total: 1.61×10^{10}	1.61×10^{10}
+/-: 0.44	0.652
FUNGI:	FUNGI:
Actinomyces	Actinomyces
Cephalosporium	Gliocladium
Cladosporium carrioni	Penicillium
Gliocladium	Streptomyces
Mucor	Verticillium
Penicillium	
Scopulariopsis	
Streptomuces	
Trichoderma	
YEAST:	YEAST:
Candida albicans	Candida albicans
Candida famata	Candida famata
Cryptococcus albicus var albi	Dandida humicola
Cryptococcus laurentii	Candida stellatoidea
Debaryomyces hansenii	Cryptococcus albidus var albi
Trichosporon beigleii	Cryptococcus gastricus
Trichosporon pullulans	Gryptococcus laurentii
	Debaromyces hansenii
BACTERIA:	BACTERIA:
Acinetobacter lwoffii	Acinetobacter lwofffii
Achromobacter biotype 2	Alcaligenes faecalis
Citrobacter freundii	Enterobacter agglomerans
Enterobacter gergoviae	Group M-4 Moraxella-like
E. coli (A-D group)	Providencia alcalifaciens
Proteus mirabilis	Providenica rettgeri
Pseudomoans aeruginosa	Providenica stuartii
Pseudomonas pseudomallei	Pseudomonas cepacia
Pseudomonas putida	Pseudomonas flourescens
Serratia marcescens	Pseudomonas pseudomallei
Bacillus	Pseudomonas species
Micrococcus	Serratia marcescens
Staphylococcus aureus	Bacillus
	Micrococcus
	Staphylococcus
	PROTOZOA:
	Euglena (flagella)
	Paramecium (ciliated, free swim.)
	Philodina (rotifer, ciliated)
	Trachelomonas (flagella)
	Vorticella (ciliated; stalked)

LIVINGSTON: 2-CHLOROPHENOL ACCLIMATED (10/8/85)

Total: 3.75×10^{10} organisms/ml
+/- : 0.167

FUNGI:
Actinomyces
Cladosporium carrioni
Cladosporium sp.
Madurella mycotomi
Scopulariopsis
Streptomyces
Penicillium

YEAST:
Candida intermedia
Cryptococcus albidus var albi
Cryptococcus gastricus
Cryptococcus laurentii
Debaromyces hansenii

BACTERIA:
Achromobacter sp. biotype 2
Acinetobacter lwoffii
Enterobacter agglomerans
Eschericia coli
Providencia stuartii
Serratia marcescens
Staphylococcus
2K-1 Pseudomonas-like

PROTOZOA:
Carchesium (branched colony, stalk, cilia)
Condylostonia (cilia)
Cyclotella (diatom)
Euglena
Paramedium (ciliated free-swimmer)
Petalomonadidae (1 flagellum)
Triangulomonas rigida (2 flag)
Ulothrix (algae)
Vorticella

PASSAIC VALLEY SEWERAGE COMMISSIONERS: Fresh (2/26/85)

Total: 2.344×10^{10} organisms/ml
+/- : 0.818

FUNGI:
Actinomyces
Aspergillus niger
Cahetomium
Cladosporium sp.
Epidermophyton floccosum
Gliocladium
Helminthosporium
Microsporum nanum
Monilia sitophila
Penicillium
Scopulariopsis
Streptomyces
Trichophyton schoenleini

YEAST:
Candida albicans
Candida intermedia
Candida stellatoidea
Candida tropicalis
Candida zeylanoides
Cryptococcus albidus var albi
Prototheca zopfii
Saccharomyces cerevisae
Trichosporon beigleii

BACTERIA:
Achromobacter species biotype 2
Acinetobacter lwoffii
Citrobacter freundii
Enterobacter agglomerans
pasteurella ureae
Pseudomonas cepacia
Pseudomonas maltophilia
Pseudomonas putida
Shigella dysenteriae
Shigella slexneri
Bacillus
Micrococcus
Staphylococcus

PROTOZOA:
Carchesium
Cymbella (chrysophyta-dinoflag.)
Euglean
Gomphonema (chrysophyta-dinoflag.)
Heteronmea (2 flag.)
Pandorina (colony, 4 flag.)
Paramecium
Phacus (free swim; 1 flag.)
Protospongia (1 flag.)
synura
Vorticella

PVSC: PHENOL ACCLIMATED (7/10/85)

FUNGI:
Aspergillus
Cladosporium carrioni
Curvularia
Epidermophyton floccosum
Fonsecaea pedrosoi
Gliocladium
Microsporum distrotum
Microsporum vanbreuseghemii
Mucor
Penicillium
Rhizopus
Streptomyces
Trichophyton schoenleini
Trichophyton tonsurans
Trichophyton virrucosum

YEAST:
Candida albicans
Cadida famata
Candida humicola
Cryptococcus albidus var albi
Cryptococcus laurentii
Cryptococcus neoformans
Debaromyces hansenii
Trichosporon beigleii
Trichosporon pullulans

BACTERIA:
Acinetobacter lwoffii
Enterobacter agglomerans
Entrobacter gergoviae
E. coli
Group M-4 Moraxella-like
Group 2K-1 Pseudomonas-like
Moraxella species
Pseudomonas cepacia
Pseudomonas flourescens
Pseudomonas marcescens
Pseudomonas species
Serratia marcescens
Bacillus
Micrococcus
Staphylo9coccus

PVSC: 2,6-DICHLOROPHENOL (1/25/86)	(2/17/86)
Total: 2.046×10^{10} organisms/ml	2.18×10^{10}
+/-: 0.9375	0.517
FUNGI:	FUNGI:
Geotrichum	Gliocladium
Gliocladium	Monosproium spiospermum
Penicillium	Penicillium
Trichoderma	Sepedonium sp
	Trichoderma sp
	Trichophyton schoenleini
	Trichophyton tonsurans
YEAST:	YEAST:
Candida albicans	Candida famata
Candida famata	Cryptococcus albidus var albi
Candida glabrata	Cryptococcus laurentii
Candida humicola	Hansenula anomala
Cryptococcus albidus var albi	Trichosporon beigleii
Cryptococcus gastricus	
Cryptococcus laurentii	
Cryptococcus neoformans	
Saccharomyces Cerevisae	
Trichosporon beigleii	
BACTERIA:	BACTERIA:
Acinetobacter lwoffii	Acinetobacter lwoffii
Alcaligenes faecalis	Alcaligenes faecalis
Enterobacter agglomerans	Enterobacter agglomerans
Group M-4 Moraxella-like	Group 2K-1 Pseudomonas-like
Providenica stuartii	Pasteurella ureae
Pseudomonas cepacia	Providencia stuartii
Pseudomonas species	Pseudomonas cepacia
Serratia Marcescens	Shigella sonnei
(Pseudomonas maltophilia)	(Achromobacter sp biotype 2)
(Pseudomonas aeruginosa)	(Pseudomonas aeruingoa)
Micrococcus	Bacillus
Staphylococcus aureus	Micrococcus
Streptococcus	Staphylococcus
	Staphylococcus aureus

PVSC: 2,6-DICHLOROPHENOL (2/17/86)

PROTOZOA:

*Chlamydomonas (2 flagella)
Chlorogonium (solitary cells, 2 flag.)
Cymbella
Euglena
Pandorina (spherical colony; 1,2,4 flag.)
Trachelomonas
Trichamoeba
Vorticella