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ABSTRACT

Microbial Characteristics of an
Activated Sludge and Three Commercial
Preparations After Exposure
to Phenolics

Laurie Gneiding
Master of Science, 1984

Thesis directed by:
Dr. Gordon A. Lewandowski
Professor of Chemical Engineering

The biological characteristics of four microbial mixtures were examined after successive exposure to phenol and 2-chlorophenol at room temperature. One of the microbial mixtures was an activated sludge from the Livingston, NJ municipal wastewater treatment plant. The other three mixtures were commercial preparations sold by Polybac Corp.(Hydrobac), Sybron Corp.(BI-CHEM DC 1006/1007), and General Environmental Sciences(LLMO).

The characteristics examined were relative counts and species of gram negative/gram positive bacteria, yeast and fungi, protozoa, and higher organisms. For the gram negative bacteria and yeasts, culturing techniques and diagnostic tubes were used to determine the predominant species. Biochemical tests were done on gram positive bacteria. Molds, protozoa, and higher organisms were identified morphologically under a light microscope.

These characteristics were determined for the freshly prepared microbial mixtures, after exposure to 100 ppm phenol for 10 days, and after further exposure to 20 ppm 2-chlorophenol for 10 more days(only the fresh LLMO was characterized).

As expected, the municipal mixed liquor exhibited a much

wider ecological diversity than any of the commercial preparations. Furthermore, although numbers of organisms were reduced after phenolic exposure, the municipal mixed liquor continued to maintain surprising diversity.

Microbial Characteristics of an Activated Sludge and Three Commercial Preparations After Exposure to Phenolics

by
Laurie Gneiding

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 Engineering/Toxicology
1984

APPROVAL SHEET

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Activated Sludge and Three Commercial
Preparations After Exposure
to Phenolics

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ACKNOWLEDGEMENTS

The author wishes to gratefully acknowledge the assistance and support of Dr. Gordon Lewandowski, Dr. Adesh Saxena, and Dr. Mohendra Kothary in the preparation of this paper. Further thanks go to Jeff, Sal, and Nigel for making it fun.

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A. General Degradation

Activated sludge contains a wide variety of organisms and a large number of bacteria(on the order of 10^{-11}/cc). Most of the bacteria have been reported to be gram negative heterotrophs(12). Many of the organisms are tolerant and/or able to biodegrade a broad spectrum of pollutants. Biodegradation has been previously studied with heterogeneous populations of bacteria as well as with single pure strains.

Czekalowski and Skarzynski(11) in 1948, found an Achromobacter strain that thrived on 100 ppm phenol as a sole carbon source.

Davey and Turner(13)attempted to grow six strains of Pseudomonas on 100 ppm phenol. Three of the six strains were able to utilize phenol, but the other three were not. The three phenol-utilizing strains were similar to Pseudomonas fluorescens while the others were similar to Pseudomonas aeruginosa.

Tabak et. al.(38)found that Pseudomonas predominated in phenol and chlorophenol degradation, with occasional appearances by Flavobacterium and Achromobacter. Xanthomonas was unable to degrade phenol or chlorophenol, but was seen in nitrophenol and alkylphenol degradation. Phenol, at a concentration of 300 ppm, was 95% degraded in 1-2 days, whereas m-chlorophenol(150 ppm) and p-chlorophenol(300 ppm)took 3-6 days to be 95% degraded.

Dunn(15)found that Pseudomonas putida could grow on 465 ppm phenol, but was unable to grow at a concentration of 11625 ppm phenol.

Radhakrishnan and Ray(33)grew Bacillus cereus on phenol

concentrations up to 1000 ppm.

B. Effects of Temperature on Degradation

Vela and Ralston(42)established that phenol degradation was unaffected by temperature changes from 10-24^{o}C using a mixed microbial population from a wastewater treatment facility. Bacterial counts were not affected. However, the number of microorganisms capable of degrading phenol diminished as a function of temperature. Pseudomonas sp. and Pseudomonas fluorescens were the predominant phenol degraders when exposed to 100 ppm daily.

C. Population Studies During Biodegradation

Population shift studies have been done for organisms exposed to phenol and 2-chlorophenol.

Radhakrishnan and Ray(33)studied activated sludge exposure to phenol. A steady-state population among protozoa, fungi, and bacteria was established during phenol exposure(concentrations ranged from 7 to 104 ppm over 112 hours)as shown in Table 1.

Davis, et.al.(14)exposed two populations to phenol. A municipal activated sludge and an industrial seed(four bacterial strains, Acinetobacter, Alcaligenes, Flavobacterium, and Pseudomonas and one yeast, Rhodotorula)both had partial inhibition at 50 ppm. Although Davis interpreted his results otherwise, his data shows that the industrial seed degraded phenol more slowly than the activated sludge. After seven days, 37 ppm out of a 50ppm dose remained for the industrial seed. This is similar to

Table 1 . Changes in Microorganism Pattern with the Continuous-Flow Culture Inoculated with Waste-Water*.(33)

In-terval (hr)	Organisms			Nature of Culture	Conc. of Organisms (ppm)	Phenol (ppm)	pH
	Bacteria	Protozoa	Fungi				
0	Plentiful	Nil	Nil	Very thick	316	15	7.4
16	Present in very small number	Colpoda v. active in large nos.	Septated form, v. lg. no.	Coagulated very thin	-	104	6.7
40	Bacteria in aggregation	Reduced in number	Large numbers	Comparatively thick but not as much as original	-	10	7.3
64	Increasing in numbers	Reduced	Reduced	Quite opaque and flocculent	-	7	7.3
88	Relatively high number	Relatively small number	Rel. sm. no.	Quite opaque and flocculent	-	7	7.3
112+	Relatively high number	Relatively small number	Rel. sm. no.	Quite opaque and flocculent	292	7	7.3

* Dilution rate 0.062/hr
\+ Steady state and balanced flora

findings from the present study in which improved results were obtained with a more heterogeneous population.

Phenol and 2-chlorophenol were studied by Baker and Mayfield(4)using soil microorganisms. The phenols were added at an initial concentration of 100 µg/ml and were rapidly degraded(12-24 hours for ≥70% decrease). Organism counts increased with phenol and 2-chlorophenol under aerobic conditions(as shown in Figure 1). The rod-shaped microorganisms would not degrade phenol or chlorophenols under anaerobic conditions.

Baker, Mayfield, and Inniss(5)also studied chlorophenol degradation at low temperatures using soil, sediment, and water microorganisms. Complete biodegradation of 2-chlorophenol was achieved in 10-15 days at 20°C. Again, the numbers of microorganisms increased during aerobic degradation.

All organism counts in my research decreased with exposure to phenol and 2-chlorophenol.

D. Biodegradation Pathways

The metabolic pathways of phenol and 2-chlorophenol have been elucidated for certain organisms(see Figures2,3,and 4).

Thirty-one strains of bacteria from the St. Lawrence River were found to degrade phenols according to Visser, et.al.(43)(as shown in Table 2). Strains belonging to Achromobacter, Azotobacter, Bacillus, Brevibacterium, Clostridium, Flavobacterium, Micrococcus, Pseudomonas, and Sarcina were able to tolerate phenol concentrations up to 2400 ppm after adaptation. The total counts of bacteria per milliliter were given at varying

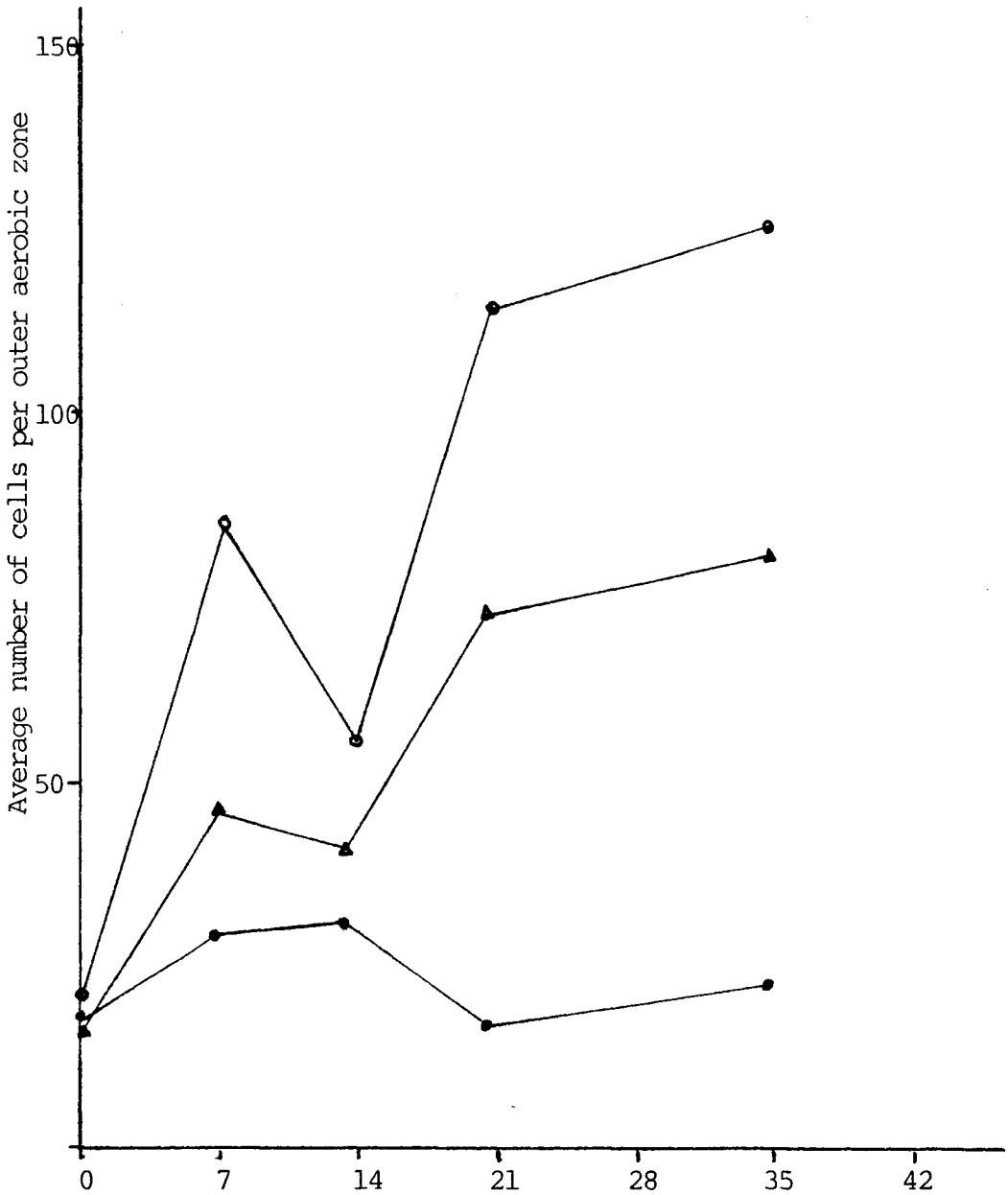


FIGURE 1. Average number of microorganisms in the outer aerobic zone of phenol and chlorophenol treated clay loam soil: Distilled water(•), phenol(▲), chlorophenol(°).(4). One zone=8 representative fields on a microscope slide.

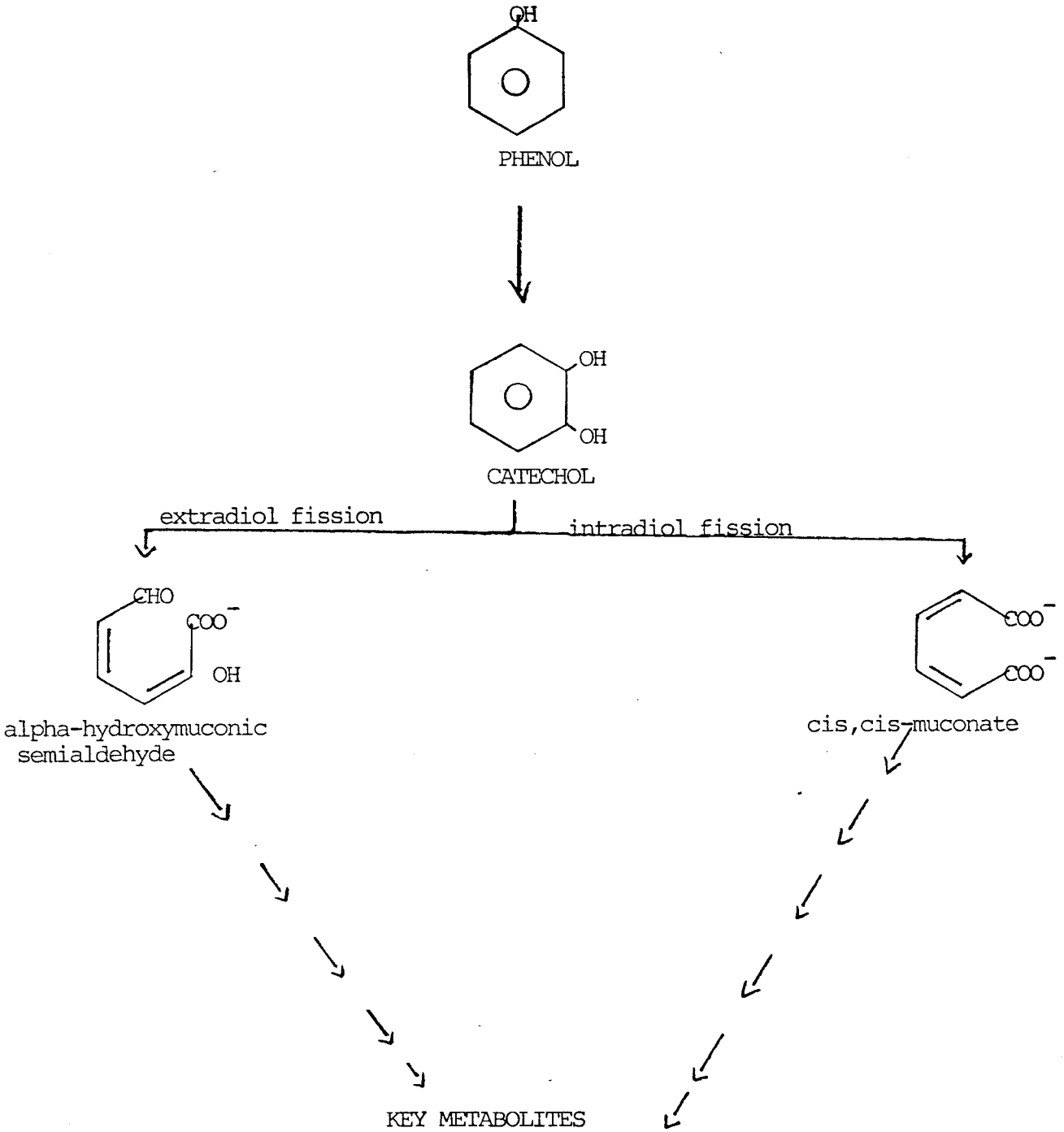


Fig. 2. Aromatic degradation in bacteria.(41).

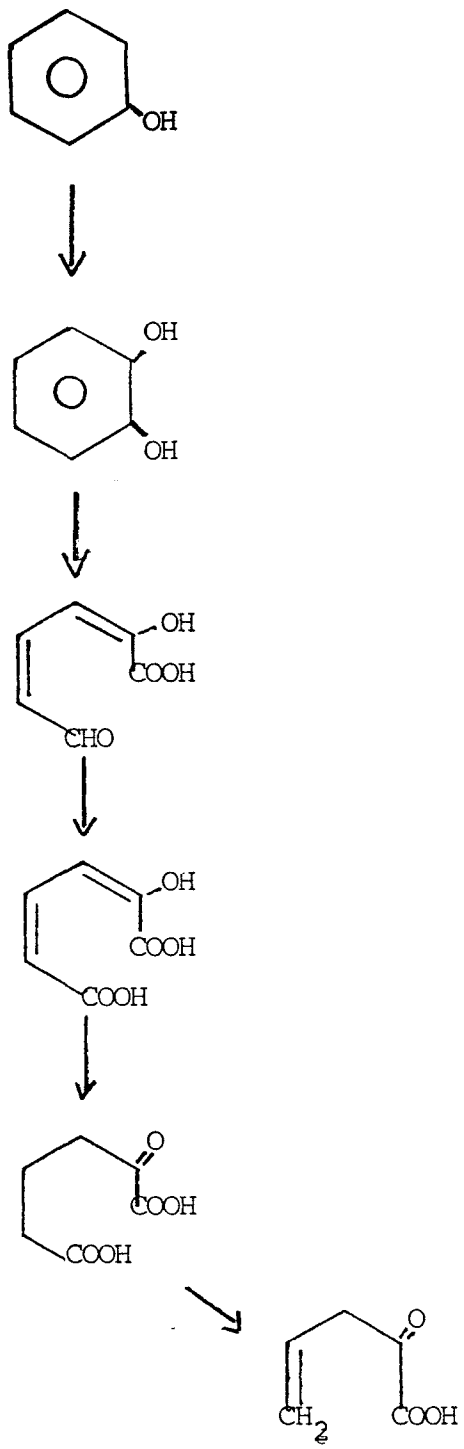


Fig. 3. Phenol acts as an inducer for the entire suite of "meta" degradation enzymes. (12)

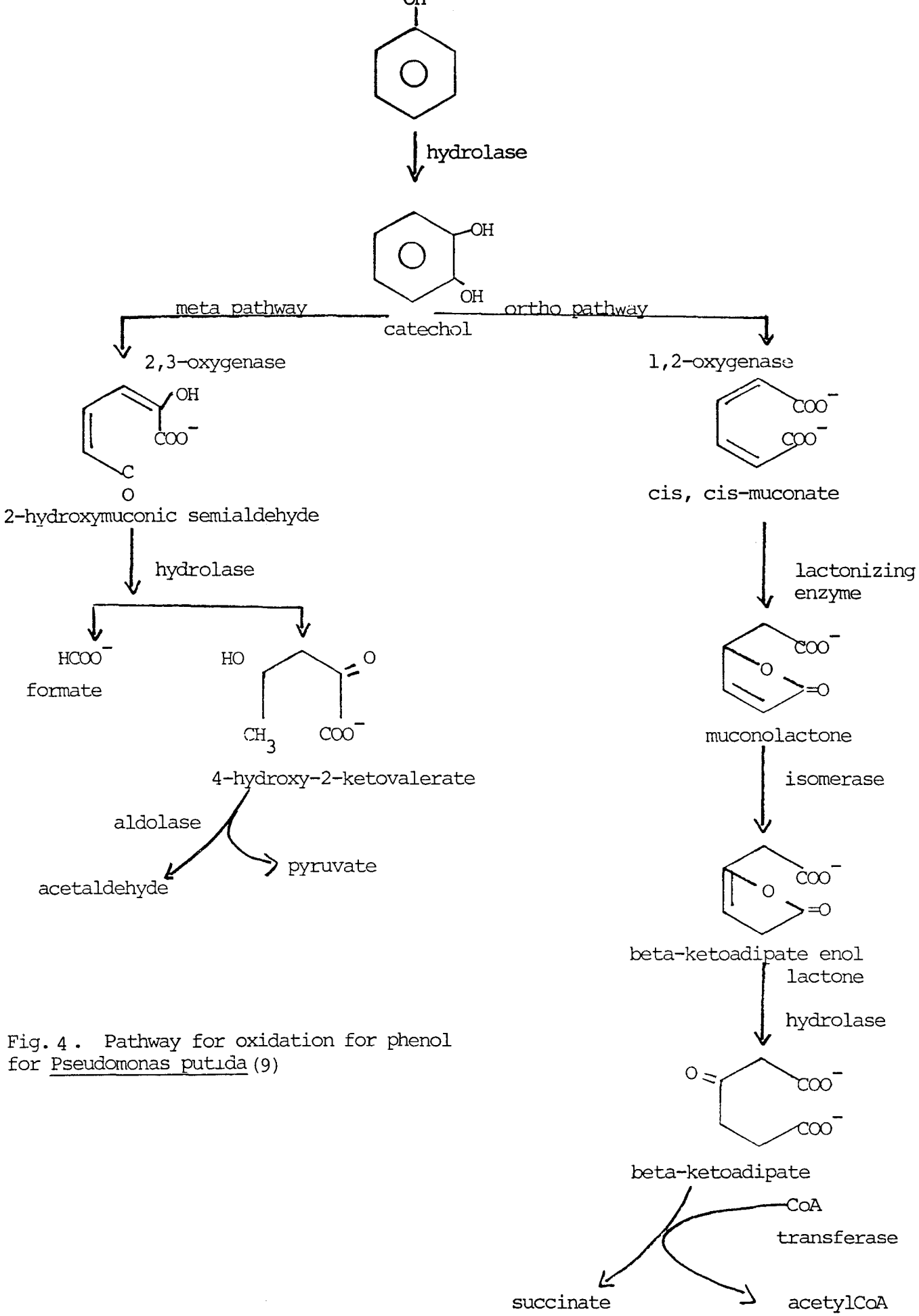


Fig. 4. Pathway for oxidation for phenol for Pseudomonas putida (9)

Table 2 . Phenol bacteria present during the summer and/or winter season in the St. Lawrence River water and the maximum phenol levels which could be tolerated.(43)

Bacterial species	Winter	Summer	Max. phenol level tolerated after adaptation(ppm)
Achromobacter eurydice		x	2400
A. cycloelastes or guttatus	x	x	1200
A. lophagus		x	1200
Arthrobacter terregens		x	1200
Azotobacter agilis		x	2400
Bacillus brevis		x	2400
B. cereus		x	600
B.circulans		x	600
Brevibacterium maris		x	2400
B.sulfureum		x	150
Brevibacterium sp.	x	x	600
Clostridium carnis or tertium		x	300
Flavobacterium aquatile or balustinum	x	x	600
F. breve or peregrinum		x	1200
F. lutescens		x	600
F. marinum	x	x	300
F. solare		x	150
Flavobacterium(marinum?)	x	x	600
Flavobacterium sp.		x	1200
Micrococcus conglomeratus		x	1200
Micrococcus(?)		x	600
Mycobacterium phlei orBrevibacterium maris		x	600
Paracolobactrum coliforme		x	2400
Pseudomonas fragi	x	x	600
Pseudomonas sp.		x	150
Pseudomonas sp.		x	300
Pseudomonas sp.		x	600
Pseudomonas sp.	x	x	1200

Table 2 continued.

Bacterial species	Winter	Summer	Max. phenol level tolerated after adaptation (ppm)
Pseudomonas sp.		x	2400
Sarcina flava		x	1200
S. hansenii*		x	2400
Unknown	x	x	--
Unknown		x	--

*Most numerous organisms

concentrations of phenol(Table 3). Most of the strains(70%)were aerobic, 27% were facultative anaerobes, and only one strain was microaerophilic. To metabolize phenol, enzymes are needed. The majority of the strains(64%)were able to produce peroxidase and 39% were able to produce phenoloxidase.

A mixture of four Pseudomonas strains, an Achromobacter, three Nocardia, a Bacillus, and Mycobacterium coeliacum were grown on phenol as a sole carbon source by Spokes and Walker(37). They found 500 ppm phenol was metabolized via the "ortho" pathway by the Nocardia and Mycobacterium strains whereas Pseudomonas and Bacillus used the "meta" pathway(see Figure 5). Complete inhibition of Achromobacter and Alcaligenes faecalis occurred on phenol. Chlorophenol was used as a carbon source by Nocardia, Mycobacterium, and Rhodotorula glutinis(yeast) via the "ortho" pathway and Pseudomonas and Bacillus oxidized catechol by "meta" fission.

Bayley and Wigmore(6)showed that mutant strains of Pseudomonas putida metabolized phenol in three ways. First, phenol is converted to catechol which then undergoes "meta" fission to 2-hydroxymuconic semialdehyde and then is metabolized to 4-oxalocrotonate. The second way is that catechol goes to 2-hydroxymuconic semialdehyde and is metabolized to 2-keto-4-pentenoate and finally, there is conversion to catechol via the "ortho"pathway. The first two pathways may function simultaneously whereas the third method is an alternative if the other two enzyme systems are blocked.

Pseudomonas sp.B13 was grown on 4-chlorophenol at a substrate concentration between 10 ppm and 14 ppm by Knackmuss and Hellwig(23). Complete inhibition occured at >1mM 4-chlorophenol

Table 3. Numbers of bacteria in the St. Lawrence River utilizing phenols at concentrations ranging from 10 ppb to 50 ppm(data in number of bacteria/ml). Three separate areas tested.(43)

	PHENOL		
Concentration	1	2	3
10 ppb	1820	1980	2060
150 ppb	1777	1644	1788
500 ppb	1741	1446	1692
15 ppm	1601	1306	1679
50 ppm	820	860	1780

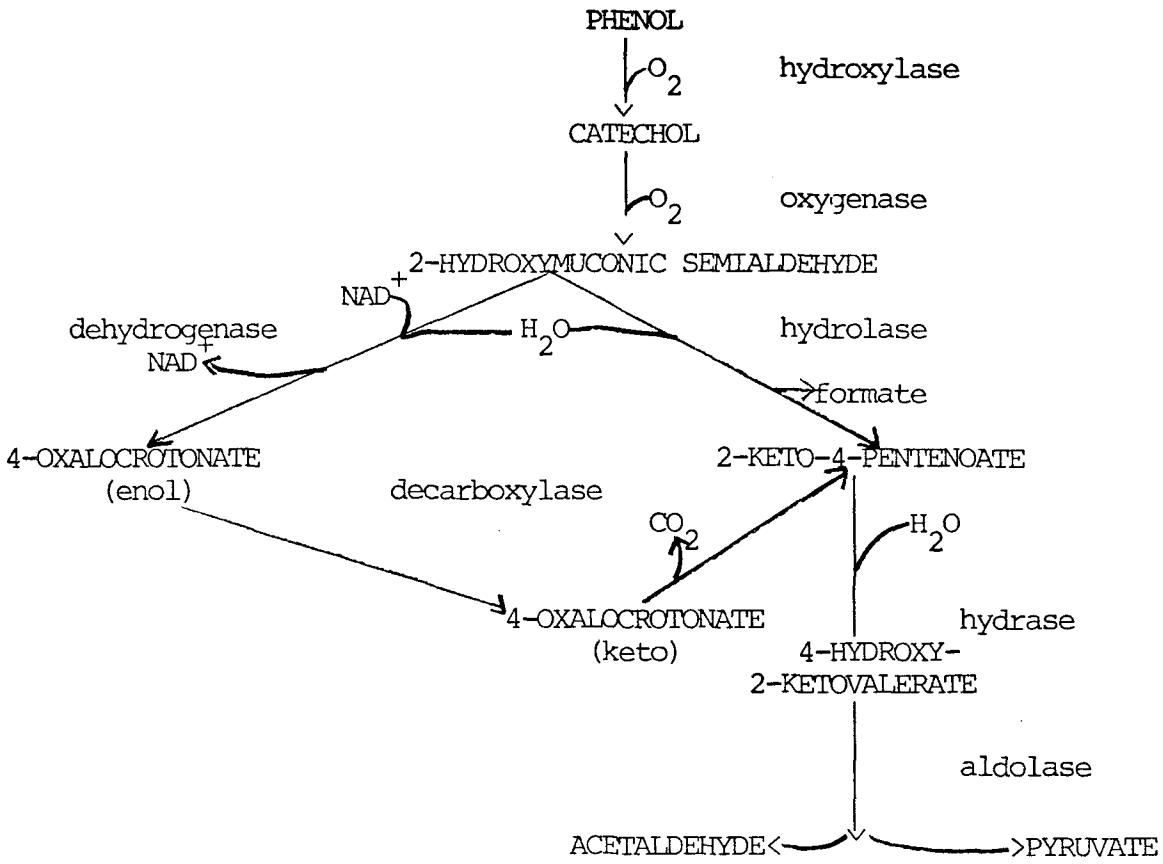


Fig. 5. The meta fission pathway for the oxidation of phenol showing the alternative modes of metabolism of 2-hydroxymuconic semialdehyde. (6,19)

whereas 70% inhibition occured at 47 ppm and 50% inhibition at 24 ppm. Rate curves are shown in Figures 6a and 6b. Alcaligenes eutrophus was found to cooxidize chlorophenols at the same rate and in the same sequence as the Pseudomonas strain. Chlorocatechol accumulated from the cooxidation of 2-chlorophenol, but no metabolites could be detected when 4-chlorophenol was cooxidized by Pseudomonas sp.B13. The pathway is shown in Figure 7.

Schwien and Schmidt(36)describe "an Alcaligenes strain which can tolerate high concentrations of phenol and which, by manual transfer of the halocatechol-degrading capacity from Pseudomonas sp.B13, acquires the ability to utilize all three isomeric chlorophenols without long adaptation procedures. The Alcaligenes sp. strain A7 was grown in a continuous culture with a mixture of phenol(470ppm)and 4-chlorophenol(190pm)for three months. Over an additional thirty day period, the 4-chlorophenol concentration was increased gradually to 560ppm. This strain degraded phenol via the "meta" pathway, whereas the constructed Alcaligenes strain A7-2 degraded phenol via the "ortho" pathway. At a concentration of 14 ppm, the 2- and 4-chlorophenol significantly inhibited the growth of Pseudomonas sp. strain B13 and 3-chlorophenol nearly prevented growth. In contrast, these three chlorophenols did not significantly affect growth of Alcaligenes sp. strain A7-2 even up to concentrations of 38ppm. It was found that 3-chlorocatechol, the metabolite formed from 2- and 3-chlorophenol, deactivated the "meta" cleaving activity. Oxidation of 4-chlorophenol yielded an accumulation of 5-chloro-2-hydroxymuconic semialdehyde. Phenol hydroxylase activity is induced by chlorophenols, with the exception of

Fig. 6A

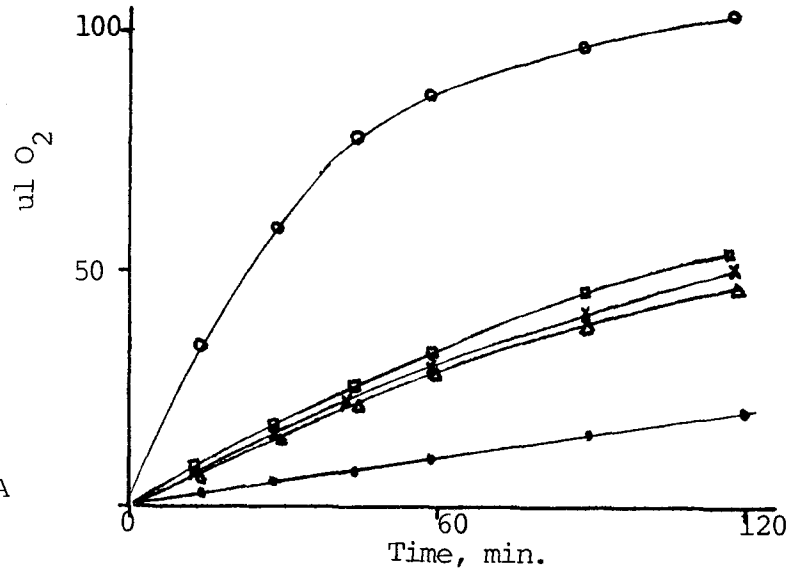


Fig. 6B

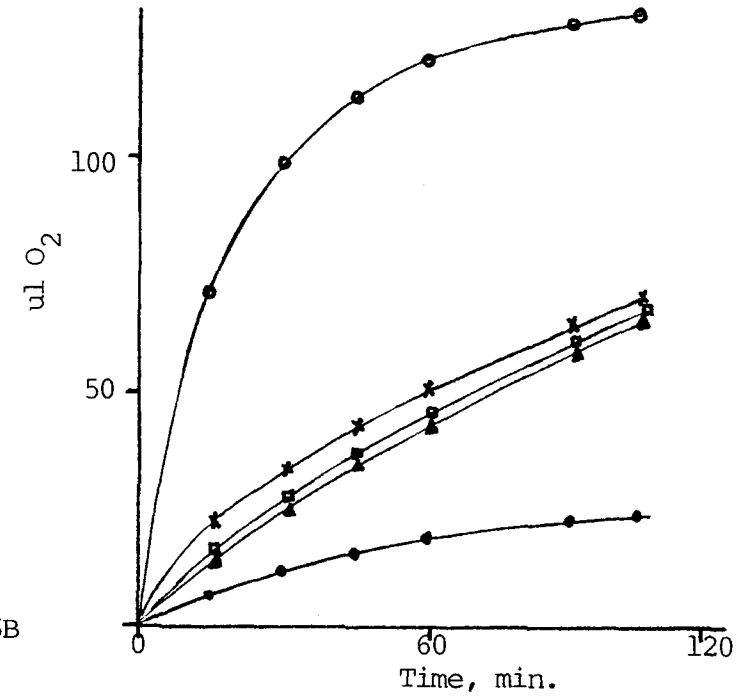


Fig. 6A. Rates of O_2 uptake by washed, phenol grown Nocardia organisms alone (•-•) and in presence of 1 μmole phenol (°-°), 1 μmole 4-chlorophenol (▫-▫), 1 μmole 3-chlorophenol (▲-▲) and 1 μmole 2-chlorophenol (x-x) (37).

Fig. 6B. Rates of O_2 uptake by washed, phenol grown Pseudomonas organisms alone (•-•) and in presence of 1 μmole phenol (o-o), 1 μmole 4-chlorophenol (▫-▫), 1 μmole 3-chlorophenol (▲-▲) and 1 μmole 2-chlorophenol (x-x) (37).

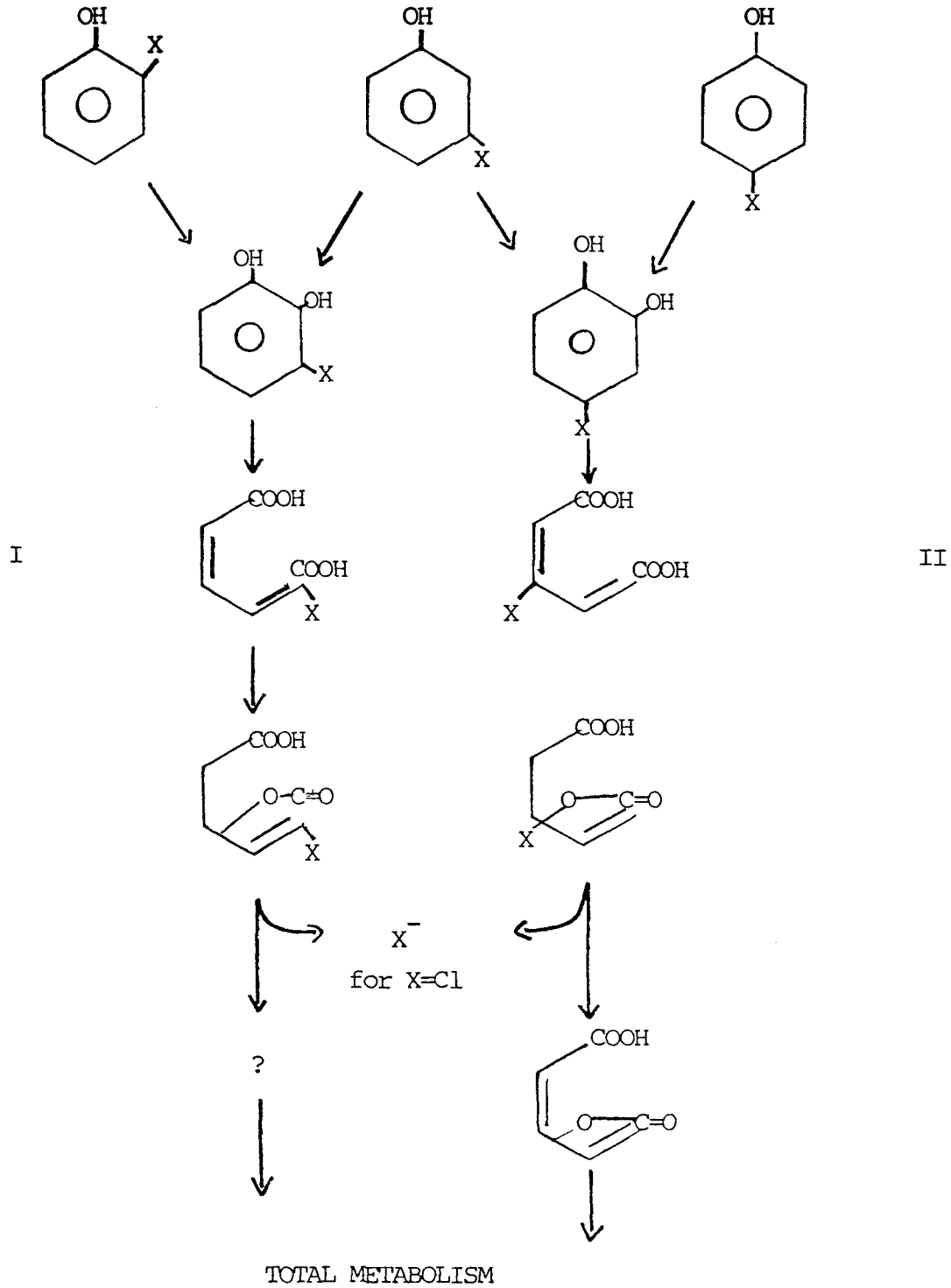


Fig. 7. Proposed pathway for the metabolism of isomeric chlorophenols (X=chlorine) and cooxidation of cresols ($X=CH_3$) by Pseudomonas sp. B13). (23)

2-chlorophenol, in Alcaligenes sp. A7-2 much faster than Pseudomonas sp. strain B13. This activity lowers the concentration of chlorophenol in cells.

Hughes, et. al.(19) proposed that Alcaligenes eutrophus degrades phenol via the "meta" cleavage pathway using two phenol hydroxylases instead of an aldehyde dehydrogenase, as shown in Figure 8.

Liu, et. al.(27) exposed a Bacillus strain to 50 ppm of various phenols, and determined the IC_{50} value (IC_{50} is the effective concentration of the toxicant causing 50% inhibition of the bacterial dehydrogenase activity). The values were 2300 ppm and 700 ppm for phenol and 2-chlorophenol respectively. Other IC50 values for chlorophenols and bromophenols are listed in Table 4.

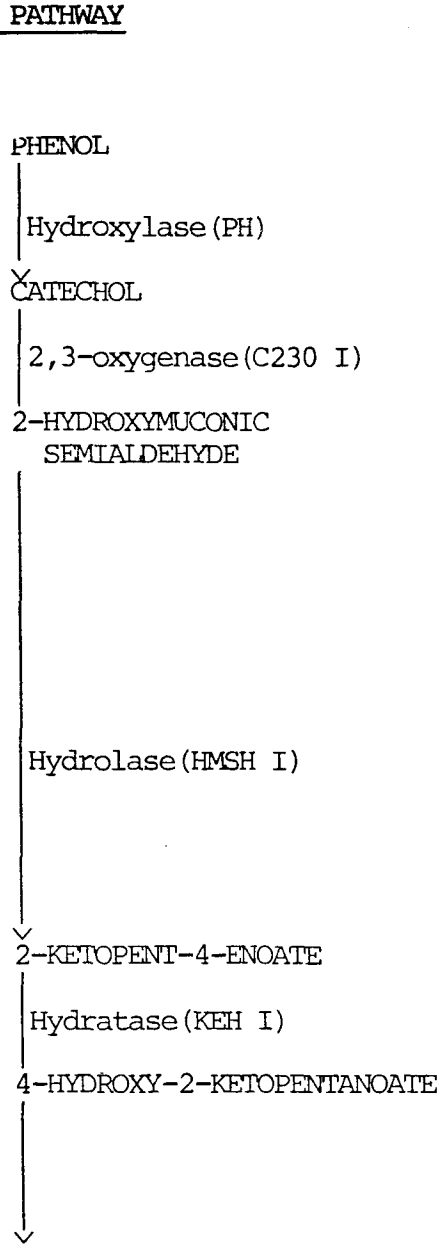


Fig. 8. Pathway for the degradation of phenol by Alcaligenes eutrophus 345.(19)

TABLE 4. IC_{50} of various chlorophenols to Bacillus sp. TL81 expressed in mg/liter. (27)

Chemicals	IC_{50} (ppm)
Phenol	2300
2-chlorophenol	700
3-chlorophenol	450
4-chlorophenol	400
2,3-dichlorophenol	130
2,4-dichlorophenol	75
2,5-dichlorophenol	85
2,6-dichlorophenol	550
3,4-dichlorophenol	52
3,5-dichlorophenol	25
2,3,4-trichlorophenol	13
2,3,5-trichlorophenol	10
2,3,6-trichlorophenol	190
2,4,5-trichlorophenol	12
2,4,6-trichlorophenol	240
3,4,5-trichlorophenol	5
2,3,4,5-tetrachlorophenol	4
2,3,5,6-tetrachlorophenol	54
pentachlorophenol	9

II. PROCEDURES

Figure 9 shows the general strategy for characterizing the microbial population.

A. Batch Feeding

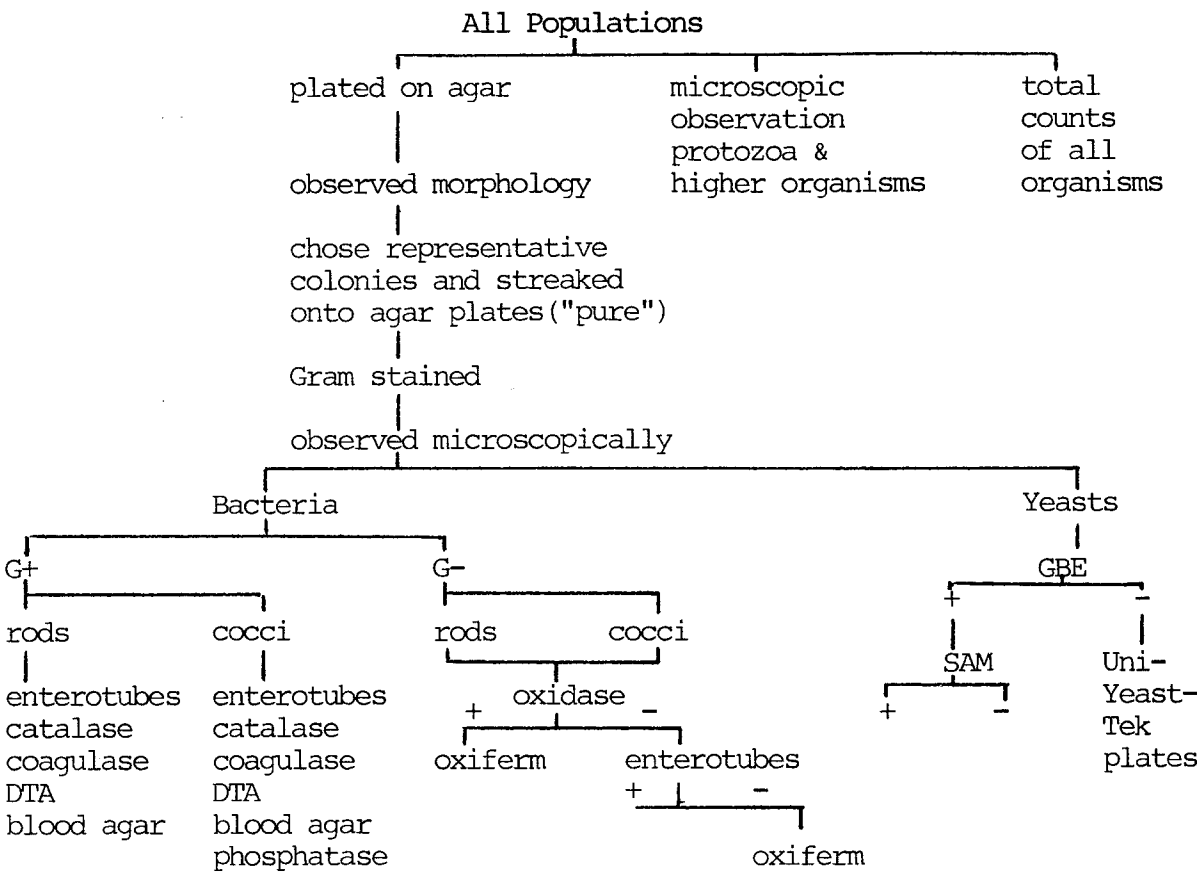
A Lucite batch reactor with an aeration rate of 500 ml/min was filled with two liters of Livingston activated sludge or one of the three commercial preparations. The preparation procedures of the commercial populations are described in Appendix A. Except for LLMO, the populations were fed 100 ppm phenol daily for 10 days, followed by 20 ppm 2-chlorophenol daily for another 10 days. The reactor was not mechanically stirred, but homogeneous biomass distribution was obtained through vigorous bubbling.

B. Characterization Techniques

A 10ml reactor sample(mixed liquor)was first shaken for about five minutes in a 20ml vial containing approximately five grams of 5mm glass beads. Then, 0.5ml was pipetted into 4.5ml of 0.1% Tween 80(aqueous)for dispersion of fungal spores. The sample was then diluted with sterile distilled water obtaining a dilution series of 10^{-1} through 10^{-12}.

Selected dilutions(10^{-1}, 10^{-3}, 10^{-5}, 10^{-10}, 10^{-11}, 10^{-12})were pipetted onto various agar plates in 0.1ml quantities. The agars used for fungal growth were Sabouraud, OXA, YM, YNB(Difco)with sucrose, Czapek(Difco)with rose bengal. The agars used for bacterial growth were plate count(Difco), floc, thio, N_2, and cellulose. After inoculation, the organisms were incubated at 20-25 ^{o}C for approximately 72 hours.

Figure 9. General Strategy of Procedures.



All procedures were done in conditions as antiseptic as possible; however, the laboratory itself was not sterile.

Gram stained slides were observed under the microscope to determine positive or negative results, and the shape of the bacteria(rods or cocci). These slides were also used to distinguish yeast colonies.

Gram positive bacterial colonies were grown on blood agar plates, subjected to catalase and coagulase tests, and inoculated onto dextrose trytone agar and phenolphthalein agar(cocci only). They were inoculated into Enterotubes(obtained from Hoffmann-LaRoche, Inc., Nutley, NJ)strictly to obtain the individual biochemical results and were not coded.

Gram negative bacteria were subjected to the oxidase test. If oxidase positive, they were inoculated in Oxiferm tubes(Hoffmann-LaRoche, Inc.)and incubated at 37°C for 48 hours, with a check at 24 hours. If oxidase positive, they were inoculated in Enterotubes and incubated at 37°C for 48 hours. Depending upon the outcome, the colonies were then coded, and the code number was read from books supplied by Hoffmann-LaRoche. Further biochemical tests were usually required, for confirmation, as outlined in the code book. Species identification was determined from the code book.

Those colonies identified as yeasts were inoculated into GBE tubes(Flow Laboratories, MacLean, VA). After 2-6 hours of incubation at 37°C, a small amount was pipetted onto a glass slide and observed, microscopically, for the presence of germ tubes. If the germ tubes were present, the yeasts were inoculated on SAM tubes(Flow Labs.)and observed for a color change in the media. If no germ tubes were present, a small amount of yeast was emulsified

in 2 ml of sterile water. Using a sterile pasteur pipet, approximately 1.5ml of solution was withdrawn and pipetted into each of the eleven well in the Uni-Yeast-Tek plates(Flow Labs.). Another small amount of yeast was streaked on the middle well for later observation of morphology.

Again, depending on the outcome, the results were coded and identification of yeast species determined from a code book supplied by Flow Labs.

Representative colonies were then streaked onto the appropriate agar and allowed to grow for an additional 24 hours at room temperature. A sample of this purified colony was then smeared onto a glass slide with a few drops of water and allowed to air dry. The smears were then Gram stained and again allowed to air dry.

Purified colonies were also examined microscopically and molds were identified by morphology.

Organism counts were obtained by placing 50 microliters of mixed liquor onto a Petroff-Haussen counting chamber. Unexposed fresh populations were diluted to 10^{-1} while the phenol and 2-chlorophenol populations were observed at full strength.

For the Hydrobac, Bichem, and LLMO preparations, it was not necessary to use all of the agars. Polybac and Bichem were inoculated on Sabouraud, OXA, plate count, floc, and cellulose agars, whereas LLMO was inoculated on plate count and floc agars.

Details of these procedures can be found in Appendix A.

fp

RESULTS

Results obtained were all for a single run.

A. Gram Negative/Gram Positive Ratios (Tables 5-8)

Table 5 to 8 shows the number of colonies for the different microbial populations on phenolic exposure. LLMO is not shown because exposure tests and counts were not run on this population.

The fresh activated sludge had approximately even numbers of gram positive and gram negative bacteria. When the sludge is exposed to phenolics, the ratio of gram positives ranges from 1.3:1 to 27:1.

By contrast, fresh Hydrobac had a high gram positive ratio which decreased on addition of phenolics. The 10^{-12} dilution had a higher count of gram negative bacteria but this may be due to contaminated dilution water.

Bichem ratios were always in favor of gram positive bacteria. After phenol exposure, no gram negative bacteria were present.

LLMO was not exposed to phenol or 2-chlorophenol. The fresh population was found to be all gram positive bacteria. This contrasts with the claim made by the manufacturer. A list provided by the manufacturer indicates the following species: Bacillus sp., Rhodopseudomonas palustris, Nitrobacter winogradskyi, Aerobacter aerogenes, Cellulomonas biazotea, Pseudomonas stutzeri, and Pseudomonas denitrificans.

In the literature gram negative species are the most

Table 5 GRAM POSITIVE VS. GRAM NEGATIVE NUMBERS OF COLONIES

Population	Agar type	G+ 10^{-10} Dilution	G-	G+ 10^{-11} Dilution	G-	G+ 10^{-12} Dilution	G-
Fresh Activated Sludge	FLOC	5	9	15	5	*	*
	PLATE COUNT	7	12	4	7	6	2
	CELLULOSE	3	0	6	0	0	1
	Total	15	21	25	12		
Phenol Activated Sludge	FLOC	24	7	200	2	61	4
	PLATE COUNT	240	6	155	3	54	5
	CELLULOSE	X	0	X	0	X	0
	Total	264	13	355	6	115	9
2-chlorophenol Activated Sludge	FLOC	50	1	79	0	59	10
	PLATE COUNT	0	5	235	2	132	11
	CELLULOSE	50	0	0	0	25	0
	Total	100	6	314	2	216	21

* no growth

TABLE 6 GRAM POSITIVE VS. GRAM NEGATIVE NUMBERS OF COLONIES

Population	Agar type	10^{-10} Dilution G+	10^{-10} Dilution G-	10^{-11} Dilution G+	10^{-11} Dilution G-	10^{-12} Dilution G+	10^{-12} Dilution G-
Bichem, Fresh	FLOC						
	PLATE COUNT						
	CELLULOSE						
	Total	—	—	—	—	—	—
Phenol Bichem	FLOC	36	0	33	0	34	0
	PLATE COUNT	90	0	50	0	71	0
	CELLULOSE	0	0	0	0	0	0
	Total	126	0	83	0	105	0
2-chlorophenol Bichem	FLOC	1	0	2	0	1	0
	PLATE COUNT	6	1	3	0	2	0
	CELLULOSE	X	0	0	0	0	0
	Total	7	1	5	0	3	0

TABLE 7. GRAM POSITIVE VS. GRAM NEGATIVE NUMBERS OF COLONIES

Population	Agar type	10^{-10} Dilution		10^{-11} Dilution		10^{-12} Dilution	
		G+	G-	G+	G-	G+	G-
Fresh Hydrobac	FLOC	53	18	350	6	101	5
	PLATE COUNT	62	10	49	4	66	2
	CELLULOSE	0	0	0	0	0	0
	Total	115	28	399	10	167	7
Phenol Hydrobac	FLOC	90	26	35	29	35	21
	PLATE COUNT	82	21	37	29	47	9
	CELLULOSE	0	2	X	X	X	X
	Total	172	49	72	58	82	30
2-chlorophenol Hydrobac	FLOC			5	70	1	116
	PLATE COUNT		30			3	333
	CELLULOSE	0	1	X	X	X	X
						4	449

TABLE 8. Ratios of gram positive vs. gram negative bacteria

	Total number of colonies for all dilutions		
	G+	G-	Ratio (G+/G-)
Fresh (Activated Sludge)	46	36	1.3:1
Phenol	734	27	27.2:1
2-chlorophenol	630	29	21.7:1
Fresh (Hydrobac)	681	45	15.1:1
Phenol	326	137	2.4:1
2-chlorophenol	1000*	550	2:1
Fresh (Bichem)	*	*	-
Phenol	314	0	-
2-chlorophenol	15	1	15:1
Fresh (LLMO)	*	-	-

*Plate overgrown but gram positive in majority.

frequently mentioned for phenolic degradation. However, a Gram positive Bacillus species was mentioned in three references(27,33,37),and Visser et.al.(43) mentioned five gram positive species in a study involving the St. Lawrence River. However, none of the literature states numbers of bacteria, or gram positive/gram negative ratios.

B. Other Microbial Counts(Table 9)

As can be seen in Table 9, the numbers of bacteria, yeast, protozoa, and the multicellular organisms decrease as phenol and 2-chlorophenol were added to the activated sludge, Bichem, and Hydrobac populations(only bacteria was present in LLMO).

Activated sludge had the most dramatic decrease in numbers when exposed to 100 ppm phenol for ten days. The bacterial numbers decreased one thousand fold whereas Bichem and Hydrobac had less than a ten fold decrease under the same conditions. However, the activated sludge was much more effective in degrading phenol and 2-chlorophenol.

In most cases, after phenol and 2-chlorophenol exposure, the yeast numbers also decreased ten fold or less.

From Table 9, protozoa numbers in the activated sludge appear to increase after exposure to phenolics. However, these data were obtained on different batches of sludge, and may only reflect variability of batches.

In the case of Bichem and Hydrobac, any organisms other than bacteria and yeasts were most likely contaminants.

Literature studies(4,5,14)indicate that organism counts

TABLE 9. Microorganism Numbers (per cubic centimeter)

ACTIVATED SLUDGE

Fresh	Phenol	2-Chlorophenol
Bacteria		
2.2×10^{11}	5×10^{7}	3.98×10^{8}
Yeast		
9.23×10^{5}	1.2×10^{6}	1.975×10^{6}
Protozoa		
1.67×10^{5}	9.6×10^{5}	5×10^{5}
Nematode		
none seen	1/50 ul	1/50 ul

BICHEM

Bacteria		
9.41×10^{8}	1.18×10^{8}	8.4×10^{7}
Yeast		
2.58×10^{6}	1.67×10^{6}	7.5×10^{5}
Protozoa		
3.76×10^{5}*	none seen	2/50 ul*

*probable contaminants

TABLE 9. Microorganisms Numbers (per cubic centimeter) (cont.)

HYDROBAC

Fresh	Phenol	2-Chlorophenol
Bacteria		
7.79×10^8	1.142×10^8	5.43×10^7
Yeast		
1.28×10^7	1.06×10^6	2×10^5
Protozoa		
none seen	none seen	none seen

increased with exposure to phenol and 2-chlorophenol. However, this is opposite to the findings of this laboratory. All populations decreased in numbers after exposure to the toxic phenolics.

C. Description of Gram Positive Bacteria(Table 10)

The majority of gram positive bacteria found in the activated sludge, Bichem, Hydrobac, and LLMO were either rod-shaped(3-4 microns long with spores),or cocci(less than one micron in diameter). The majority were, by far, the rods. A few exceptions included small two micron rods with no spores and some larger cocci. Table 10 contains characteristics of all gram positive bacteria found.

Morphologically, the rods were a white, flat, opaque colony with an irregular margin and undulate surface. These bacteria were found in all four populations. Biochemically, they reacted positively to catalase, dextrose tryptone agar(acid glucose production), glucose, lactose, phenylalanine, and urea. They had beta hemolysis on blood agar. Negative results were with adonitol, arabinose, citrate, dulcitiol, indole, lysine, ornithine, and sorbitol. A few showed the ability to utilize arabinose, citrate, or sorbitol and some produced pyomelanin. The coagulase test was usually positive. These bacteria are probably species of Bacillus.

Morphologically, the tiny cocci were a white, smooth, opaque, convex colony with a smooth margin and surface. These were seen in the Bichem population. Biochemically,

TABLE 10, GRAM POSITIVE CHARACTERISTICS

Colony description	catalase	coag-ulase	glucose (gas)	lactose	urea	phenyl-alanine	indole	arab-inose	sorb-itol	citrate	dul-citol	haem-olysis	dextrose tryptone	shape + size
*OXA AS3 yellow cont	+	−	+	−	−	±	−	−	−	−	−	β	+	rods, 3μm
SAB ASP3 wh punct sm cir	+	+	+	+	+	+	−	−	−	−	−	β	+	rods, 5μm
Czrb ASC1 punct sm cir	+	−	+(+)	−	−	−	−	−	−	+	−	β	+	rods 6μm
SAB ASC5 wh sm circ	−	+	+(+)	+	+	+	−	−	−	+	−	β	+	rods 3μm
Floc AS10 lg wh irreg und	+	+	+(+)	−	−	+	−	−	−	−	−	β	+	rods, 3μm
pc AS10 lg wh irreg und	+	−	+(+)	−	−	+	−	−	−	−	−	β	+	rods, 3μm
floc AS10 wh irreg und	+	+	+	+	+	+	−	−	−	−	−	β	+	rod, 4μm
Cellulose AS10	−	+	+(+)	+	+	+	−	−	−	−	−	β	+	cocco bacilli
pc AS10 wh irreg und	+	−	+	+	+	+	−	−	−	−	−	β	+	rods, 4μm
N_2 AS10 wh flat irreg	+	−	+	+	+	+	−	−	−	−	−	β	+	rods w/sp 4μm
N_2 AS10 wh sm	+	−	+(+)	+	+	+	−	−	−	−	−	β	+	rods
floc AS10 wh sm circ	+	−	+	+	+	+	−	−	−	+	−	β	+	rods, 6μm
floc AS10 wh flt und	+	+	+	+	+	+	−	−	−	−	−	β	+	rods 3μm w/sp.
cellulose AS10 wh flt und	+	+	+	+	+	+	−	−	−	−	−	β	+	rods w/sp 3μm
N_2 AS10 wh flt und	+	−	+	+	+	+	−	−	−	−	−	β	+	rods, 3μm
pc AS11 rugose	+	+	−	−	−	−	−	−	−	−	−	β	+	cocci 3μm phos +
cellulose AS11	+	+	+	−	+	+	−	−	−	−	−	β	+	rods, 6μm
floc AS11 wh und irr	−	−	+	−	−	+	−	−	−	−	−	β	+	rods w/sp 3μm
pc AS11 wh und	−	±	+(+)	−	−	+	−	−	−	−	−	β	+	rods, 3μm
floc AS11 wh irr und	−	±	+(+)	+	+	+	−	−	−	−	−	β	+	rods, 4μm
pc AS11 wh flt. und	−	+	+	−	−	+	−	−	−	−	−	β	+	rods, 4μm
cellulose AS11 wh irr	+	+	+	+	+	+	−	−	−	−	−	β	+	rods, 3μm
cellulose AS11 wh sm	+	−	−	+	+	+	−	−	−	−	−	β	+	cocci, phos +
pc AS11 wh flt und	+	−	+	+	+	+	−	−	−	−	−	β	+	rods, 3μm
floc AS11 wh sm circ	+	−	+	+	+	+	−	−	−	+	−	β	+	rods, 6μm
floc AS11 wh flt und	+	−	+	+	+	+	−	−	−	−	−	β	+	

TABLE 10. cont.

colony description	catalase	coagulase	glucose (gas)	lactose	urea	phenyl-alanine	indole	arabinose	sorbitol	citrate	dulcitol	haemolysis	dextrose tryptone	size + shape
ASC12 (N_2) wh flt und	+	−	+(+)	+	+	+	−	−	−	−	−	β	+	rod 3µm PVo+
cellulose AS12	+	−	+	−	−	−	−	+	−	−	−	β	+	coccobacilli 3µm
pc ASP12 wh irr und	+	−	+	−	−	+	−	−	−	−	−	β	+	rods 4µm
floc ASP12 wh transl punct	+	±	+	+	+	+	−	−	−	−	−	β	−	rods 4µm
pc AS12 radiate	+	+	−	−	−	−	−	−	−	−	−	β	+	rods 4µm
floc ASP12 wh und irr	+	−	+	+	+	+	−	−	−	−	−	β	+	rods, 4µm
floc ASC12 wh flt und	+	−	+	+	+	+	−	−	−	−	−	β	+	rods w/sp 3µm
pc ASC12 wh flt und	+	−	+	+	+	+	−	−	−	−	−	β	+	rods w/sp 3µm
SAB S3 wh sm circ	+	−	+	+	+	+	−	+	+	+	+	γ	+	lys+; orn+; ado+; phos− cocci 1µm
SAB SP1 wh sm circ	+	−	+	+	−	+	+	+	+	−	±	γ	+	orn+; phos− cocci 1µm
SAB SP1 wh transl sm circ	+	±	+(+)	+	+	+	−	+	+	+	−	γ	+	orn+; phos− cocci 1µm
OXA SP1 wh sm circ	+	−	+	−	+	+	−	−	−	+	−	γ	−	rods, 2µm
SAB SP3 wh sm circ	+	±	+	+	−	−	+	+	−	−	+	α	+	ado+; rods 2µm
SAB SC3 transl wh flt und	+	±	+(+)	−	+	+	−	−	−	−	−	β	−	rods 2µm
OXA SC3 wh transl sm	+	−	±	−	−	−	−	−	−	−	−	β	−	phos− cocci
SAB SP5 wh transl sm circ	+	−	+(+)	−	+	+	−	−	−	+	−	β	+	rods, 2µm
SAB SP5 wh sm irr	+	−	+	−	+	+	−	−	−	−	−	α	−	rods, 2µm
SAB SC5 gr wh irr	+	−	+	−	+	+	−	−	+	−	−	γ	−	PVo+; rods 2µm
pc S10 wh flt irr	+	−	+(+)	+	+	+	−	−	−	+	−	β	+	PVo+; rods w/ spores, 3µm
cellulose S10 wh flt und	+	−	+	+	+	+	−	−	−	+	−	β	+	PVo+; rods w/ spores, 3µm
pc SP10 wh flt und	+	−	+	+	+	+	−	−	−	−	−	β	+	PVo+; rods3µm
floc SP10 wh flt und	+	−	+	+	+	+	−	−	−	−	−	β	+	PVo+; rods3µm
pc SC10 wh sm circ	+	−	−	−	−	−	−	−	−	−	−	β	−	rods 1µm

TABLE 10 (cont.)

colony description	catalase	coagulase	glucose (gas)	lactose	urea	phenylalanine	indole	arabinose	sorbitol	citrate	dulcitol	haemolysis	dextrose tryptone	shape + size
floc SC10 gr wh irr	+	+	+	−	+	+	−	−	−	−	−	γ	−	rods w/sp. 3μm
pc SC10 gr wh irr	+	+	+(+)	+	+	+	−	+	+	+	−	γ	−	rods, 2μm
pc SC10 yw sm irr	+	−	+	−	−	+	−	−	−	−	−	γ	+	rods, 2μm
pc SP11 wh flt und	+	−	+	−	−	+	−	−	−	−	−	β	+	Pyo+; rods, 3μm
floc SP11 wh flt und	+	−	+	−	−	+	−	−	−	−	−	β	+	pyo+; rods, 3μm
pc SC11 yw sm irr	+	+	+(+)	−	−	+	−	−	−	−	−	β	+	rods, 3μm
floc SC11 sm wh cir	+	−	+	−	+	+	−	−	+	−	−	β	−	rods, 3μm
floc SC11 gr wh irr	+	±	+	−	+	+	−	−	+	−	−	β	−	rods, 2μm
pc SC11 wh sm	+	+	+	−	+	+	−	−	−	+	−	β	−	rods, 3μm
pc SC wh irr gr	+	+	+	−	+	+	−	−	+	+	−	β	−	rods, 3μm
pc SI2 wh irr wr	+	−	+	+	+	+	−	−	−	+	−	β	−	rods, 3μm
floc SP12 wh flt und	−	−	+	+	+	+	−	−	−	−	−	β	+	Pyo+; rods, 3μm
pc SP12 wh flt und	−	−	+	+	+	+	−	−	−	−	−	β	+	Pyo+; rods, 3μm
floc SP12 lg wh sm circ	+	+	+	−	+	+	−	−	−	−	−	γ	−	rods, 4μm
pc SC12 wh flt gr	+	−	+	+	+	+	−	−	+	+	−	β	−	rods, 3μm
pc SC12 wh sm cir	+	+	+(+)	−	+	+	−	−	−	+	−	β	−	rods, 3μm
floc SC12 wh flt und	−	+	+	−	+	+	−	−	−	+	−	β	+	rods w/sp., 4μm
SAB H5 wh flt sm	±	−	+	−	+	−	−	−	+	+	−	γ	−	rods, 3μm
SAB H5 wh pulv sm cir	+	+	+	+	−	+	−	+	−	+	−	β	−	phos−; cocci
SAB HC1 wh transl sm cir	+	±	+	−	+	+	−	+	+	+	−	β	+	rods, 2μm
SAB HC3 wh sm cir			+(+)	−	+	+	−	−	−	−	−			rods w/sp. 4μm
SAB HC5 wh transl sm cir	+	+	+	−	+	+	−	−	−	+	−	β	+	rods, 3-4μm
SAB HC5 wh flt und	+	+	+	+	+	+	−	+	+	+	−	γ	+	rods w/sp. 3μm

TABLE 10. (cont.)

Colony description	catalase	coag-ulase	glucose (gas)	lactose	urea	phenyl-alanine	indole	arab-inose	sorb-itol	citrate	dul-citol	haem-olysis	dextrose tryptone	Shape + size
SAB HC5 transl irr	+	-	+	+	+	+	—	+	+	+	—	β	+	rods, 2 µm
SAB HC5 wh sm cir	+	-	+	+	+	+	—	—	-	+	-	β	+	rods, 3 µm
SAB HP5 wh irr gr	+	-	+	—	—	—		—	+	—	—	β	-	phos-; cocci in 3s 1 µm
SAB HP5 yw circ gr	-	+	-	-	-	—	—	—	—	-	-	β	+	phos-; cocci 1 µm
OXA HP5 yw cir sm	—	+	—	—	—	—	-	-	-	-	-	β	+	phos-; cocci 1 µm
SAB HP1 wh tr. sm	+	+	+(+)	+	+	+	+	+	+	+	+	β	+	lyst; orn+; pyo+; phos-; cocci 2 µm
SAB HP3 wh sm cir	-	-	-	—	—	—		—	—	—	—	β	—	rods w/sp 3 µm
floc H10 wh flt und	+	-	+	+	+	+	-	-	-	+	-	β	+	rods, 3 µm
pc H10 wh flt und	+	-	+(+)	+	+	+	—	-	-	±	-	β	+	rods, 3 µm
H11 wh flt und	-	±	+	+	+	+	—	-	-	-	-	β	+	Pyo+; rods, 3 µm
pc H12 wh sm circ	+		+	—	+	+	—	+	+	+	-			rods, 4 µm
floc H12 wh flt und	-	-	+	+	+	+	—	—	—	-	-	β	+	rods, 3 µm
pc H12 wh sm circ	+	+	+(+)	+	+	+	-	-	-	-	-	β	+	rods, 2 µm
pc HC10 wh flt und	+	-	+	+	+	+	—	—	—	+	-	β	+	rods w/sp 4 µm
pc HC10 wh sm cir	+	+	+	+	+	+	—	+	+	+	-	β	—	rods, 3 µm
floc HC10 wh flt irr und	+	-	+(+)	+	+	+	—	-	—	+	-	β	+	Pyo+; rods w/sp 4 µm
floc HC10 wh sm cir	+	+	+	—	+	+	—	—	—	—	-	β	+	rods 2 µm
pc HC11 wh flt und	+	-	+	—	-	—	-	-	-	-	-	β	—	rods w/sp 4 µm
floc HC11 wh flt und	+	-	+	—	-	+	—	-	-	+	-	β	+	rods w/sp. 4 µm
floc HC12 wh flt irr	+	-	+	+	+	+	—	-	-	+	-	β	+	Pyo+; rods w/sp 4 µm
pc HC12 wh flt und	+	-	+(+)	—	+	+	—	-	-	-	-	β	+	rods w/sp. 4 µm
pc HP12 clear wh irr	+	-	+	+	—	-	—	-	+	-	-	β	-	phos-; cocci in chains, 1 µm
floc HP10 wh flt und	+	±	+	+	+	—	-	—	—	—	-	β	+	rods w/sp., 4 µm
pc HP11 wh flt und	+	—	+	+	—	—	—	—	—	—	—	β	+	rods w/sp, 4 µm

TABLE 10. (cont.)

colony description	catalase	coagulase	glucose (gas)	lactose	urea	phenyl-alanine	indole	arabinose	sorbitol	citrate	dulcitol	haemolysis	dextrose tryptone	shape + size
cellulose HW wh flt und	+	±	+	+	+	+	−	−	−	+	−	β		Pyo+;
pc HP11 lg wh gr	+	±	+	−	−	−	−	−	+	−	−	β	+	phos−; cocci in chains, 1um
floc HP11 wh flt und	+	+	+	+	−	−	−	−	−	−	−	β	+	rods w/sp 4um
floc HP12 wh flt und	+	+	+	−	+	+	−	−	−	−	−	β	+	rods w/sp. 4um
pc HP10 wh clearin	+	+	+	−	−	−	−	+	+	−	−	β	+	phos−; cocci in chains; 1um
pc HP10 wh flt und	+	+	+(+)	+	+	+	−	−	−	−	−	β	+	rods w/sp 4um
pc HP12 wh flt und	+	−	+	+	+	+		−	−	−	−	β	+	rods w/sp 4um
BACTISOLV			+	+	+	+	−	−	−	−	−	β	+	rods, 3um w/sp.

CODES: *AS = activated sludge
ASP = phenol acclimated A/S
ASC = 2-chlorophenol acclimated A/s
S = Bichem
SP = phenol acclimated Bichem
SC = 2-chlorophenol acclimated Bichem

H = Hydrobac
HP = phenol acclimated Hydrobac
HC = 2-phenol acclimated Hydrobac

agar : population : dilution factor

they utilized arabinose, catalase, citrate, dextrose tryptone, glucose, lactose, ornithine, phenylalanine, and sorbitol. Negative results were obtained with indole and phosphatase. No hemolysis(gamma)was seen on blood agar. Coagulase results were mixed. These organisms may be Micrococcus.

In addition, there were larger cocci found in Hydrobac which were whitish, transluscent colonies with an irregular margin and a grainy surface. Biochemically, positive results were achieved with catalase, citrate, glucose, and sorbitol. Negative results were obtained with arabinose, citrate, dulcitol, indole, lactose, phenylalanine, phosphatase, and urea. Blood agar showed beta-hemolysis. Coagulase results were mixed. I was unable to classify these organisms.

Another group of cocci, found in the Livingston activated sludge was phosphatase positive indicating the possibility of Staphylococcus aureus. These organisms showed positive results with catalase, coagulase, dextrose tryptone agar, and phosphatase. On cellulose agar, positive results occured on lactose, phenylalanine, and urea. There were other activated sludge bacteria that grew on plate count agar, but showed negative results with the above agars.

Bacillus species have been mentioned in the literature as degraders of phenolics(15,33,38,43)as well as Micrococcus(43), but Staphylococcus aureus was not mentioned.

D. Description of Gram Negative Bacteria(Tables 11-13)

The microbial population of activated sludge is much

more diverse than that of Hydrobac, Bichem, or LLMO. A variety of enteric bacteria were found in the activated sludge, as well as species of Achromobacter, Acinetobacter, Aeromonas, Alcaligenes, and Pseudomonas as shown in Tables 11 to 13.

As the sludge was exposed to phenol and 2-chlorophenol, only a few of the enteric bacteria remained. The dominating genera were the flagellated Pseudomonas and Alcaligenes. Visser, et. al(43) also found these genera in a phenol acclimated mixed liquor, as well as Flavobacterium(43).

Bichem had a limited variety of gram negative species. These were only pseudomonads that were persistent throughout exposure to phenol and 2-chlorophenol.

Hydrobac had a more varied population than Bichem. Pseudomonas, Acinetobacter, Aeromonas, and Xanthomonas were observed.

No gram negative species were found in the LLMO populations though the manufacturer claims otherwise. This may be due to the fact that these bacteria were from the last 100 ml of the bottle and were over six months old.

No literature studies were found concerning microbial characteristics of commercial preparations during phenolic exposure.

E. Description of Fungi(Tables 14-16)

Fresh activated sludge has a spectrum of fungal species(17). Those identified in the present study as major species in the Livingston sludge are shown in Table 14.

TABLE 11. Fresh Bacterial Populations

Livingston A/S	Bichem	Hydrobac	LLMO
Achromobacter xylosoxidans	Pseudomonas sp.	Acinetobacter lwoffi	Bacillus
Acinetobacter lwoffi	Pseudomonas aeruginosa	Pseudomonas sp.	
Aeromonas hydrophila	Gram positive cocci	Pseudomonas cepacia	
Alcaligenes faecalis	Bacillus	Pseudomonas putida	
Enterobacter agglomerans		Bacillus	
Escherischia coli		Gram positive cocci	
Pasturella ureae		Group 5E Pseudomonas-like	
Pseudomonas sp.		Aeromonas hydrophila	
Pseudomonas sp.		Xanthomonas	
Pseudomonas vesicularis			
Serratia liquefaciens			
Xanthomonas sp.			
Bacillus sp.			
Gram positive cocci			

TABLE 12. Phenol Acclimated Bacterial* Populations

Livingston A/S	Bichem	Hydrobac
Alcaligenes faecalis	Pseudomonas sp.	Pseudomonas sp.
Enterobacter agglomerans	Pseudomonas fluorescens	Pseudomonas cepacia
Enterobacter cloaceae	Bacillus	Pseudomonas vesicularis
Pseudomonas sp.	Gram positive cocci	Acinetobacter lwoffii
Pseudomonas aeruginosa		Aeromonas sp.
Pseudomonas cepacia		Bacillus
Serratia liquefaciens		Gram positive cocci
Serratia rubidea		
Xanthomonas		
Gram positive cocci		

*LLMO was not subjected to microbial analysis after phenol acclimation.

TABLE 13. 2-Chlorophenol Acclimated Bacterial* Populations

Livinston A/S	Bichem	Hydrobac
Achromobacter	Pseudomonas sp.	Pseudomonas sp.
Acinetobacter lwoffii	Pseudomonas fluorescens	Pseudomonas aeruginosa
Alcaligenes faecalis	Bacillus	Pseudomonas putida
Pseudomonas aeruginosa		Xanthomonas
Pseudomonas cepacia		
Pseudomonas fluorescens		
Pseudomonas stutzeri		
Xanthomonas		
Bacillus		
Gram positive cocci		

*LLMO was not subjected to microbial analysis after 2-chlorophenol acclimation.

TABLE 14. Fresh Fungal Populations*

Livingston A/S	Bichem	Hydrobac
Candida albicans	Aspergillus niger	Candida albicans
Candida intermedia	Candida sp.	Penicillium sp.
Candida stellatoidea	Candida stellatoidea	
Cryptococcus unigluttulatus	Penicillium sp.	
Debaromyces hansenii	Trichosporon beigleii	
Geotrichum sp.		
Penicillium sp.		
Saccharomyces cerevisiae		
Streptomyces sp.		
Trichophyton verrucosum		

*LLMO did not contain any fungal species

TABLE 15. Phenol Acclimated Fungal Populations

Livingston A/S	Bichem	Hydrobac
Aspergillus sp.*	Candida albicans	Candida albicans
Candida albicans	Penicillium sp.	Penicillium sp.
Candida stellatoidea	Rhizopus sp.	Rhizopus sp.
Cladosporium wernecki*	Trichosporon beigleii	
Debaromyces hansenii	Cryptococcus unigluttulatus*	
Penicillium sp.		
Rhizopus sp.*		
Streptomyces sp.		
Trichoderma sp.*		
Trichosporon beigleii		
Trichosporon inkin		

*possible contaminants

TABLE 16. 2-Chlorophenol Acclimated Fungal*Populations

Livingston A/S	Bichem	Hydrobac
Candida albicans	Candida albicans	Candida albicans
Candida famata	Penicillium sp.	Candida humicola
Cryptococcus unigluttulatus	Rhizopus sp.	Cryptococcus unigluttulatus
Penicillium sp.	Trichosporon beigleii	Penicillium sp.

* LLMO was not subjected to microbial analysis after 2-chlorophenol acclimation.

Many fungal species are capable of degrading phenolics. Varga and Neujahr(41)grew Trichosporon cutaneum and Candida tropicalis on 30 ppm phenol, and the yeasts were able to metabolize it to catechol and ultimately to a cis, cis-muconate. Four mycelial fungi, although not identified, also metabolized phenol in the same manner as the yeast. The degradation pathway is shown in Figure 2.

I found that Trichosporon and Candida were able to grow on 100 ppm phenol and 20 ppm 2-chlorophenol. Degradation products were not studied in my research

Ristanovic' et.al.(34)achieved 84% sporulation with salt and fresh water fungal strains on 500 ppm phenol. Fresh water species included Alernaria, Aspergillus, Botryotrichum, Cladosporium, Penicillium, Phoma, Trichoderma, and Trichothecium. A complete list and the percent degradation is listed in Table 21. I also found Aspergillus, Cladosporium, Penicillium, and Trichoderma to be phenol tolerant.

Antai and Crawford(2)demonstrated, for the first time, Streptomyces setonii metabolizing phenol. Cultures were grown at 45^{o}C at a concentration of 1000 ppm phenol with mineral salts. This degradation pathway is similar to the findings of Varga and Neujahr. Streptomyces setonii degraded phenol through catechol and to cis, cis-muconic acid via catechol-1,2-dioxygenase. Subsequent metabolism was presumably through the catechol branch of the beta-ketoadipate pathway(Figure 2).

Kwasniewska and Kaiser(24)tested oxidative and fermentative yeasts on selected phenols. Species of Pichia,

Torulopsis, Rhodotorula, and Saccharomyces and Rhodotorula rubra were exposed to phenol in concentrations of 10-100 ppm and 4-chlorophenol in concentrations of 5-150 ppm. It was found that the oxidative strains, Rhodotorula and Torulopsis were more sensitive than the fermentative species of Saccharomyces and Pichia. The fermentative yeasts have a different complex of enzymes that make them more resistant to toxic materials. Oxidative yeasts have carotenoid lipophilic membranes that allow diffusion of lipophilic contaminants, such as phenol, through the cell wall. Phenols modify surface activities and therefore can affect protein denaturation, enzyme deactivation, and disruption of cell membranes. Chlorophenols pose problems for the oxidative Rhodotorula rubra but not for Pichia. Concentrations of 100 ppm of p-chlorophenol inhibited 19-70% of Rhodotorula rubra as opposed to 12-15% of Pichia.

Saccharomyces and Rhodotorula were found in the fresh Livingston activated sludge but not in any phenolic populations.

According to Cserjesi(9), Aspergillus and Penicillium were able to grow on pentachlorophenol(PCP)up to 20ppm. Some growth of Aspergillus was seen on sodium pentachlorophenoate(NaPCP) up to 640 ppm, and with Penicillium up to 320 ppm. Trichoderma was also reported to degrade PCP.

My research found that Penicillium was the most persistent mycelial fungi throughout the phenol and 2-chlorophenol runs. The predominant yeast genera were Candida and Trichosporon.

Bichem and Hydrobac may have an indigenous fungal population, but those detected may also be contaminants. Candida is the predominant genus in these two commercial populations.

F. Description of Protozoa(Table 17-19)

A diverse population of protozoa inhabits the Livingston activated sludge. Curds(10) lists 228 species of protozoa that have been identified in activated sludge. The most frequently found are listed in Table 22. A list of the species found in my research can be found in Table 17-19. These were also reported elsewhere(10,33). The density in the Curds study was reported at 50,000 cells/ml which is roughly the same magnitude found in my studies. The ciliates are the predominant class over the ameboids and flagellates in Curds and in my research.

Hydrobac and Bichem should not have a protozoa population. Those few observed, were most likely contaminants.

Honig et.al.(18) tested toxicity of phenol using Chilomonas paramecium. It was found that this protozoan could withstand phenol insults up to 95 ppm at 20°C. The lowest concentration at which all organisms died within ten minutes(the lethal concentration)was 1500 ppm, but the highest concentration at which some organisms survived after three hours(the tolerated concentration)was 560 ppm.

Ruthven and Cairns(35) found that many protozoa were phenol tolerant. Ameba proteus, Blepharisma, Chilomonas, Euglena, Paramecium multimicronucleatum, Peranema, and Tetrahymena were more tolerant of phenol than Daphnia.

Radhakrishnan and Sinha Ray(33)exposed activated sludge to phenol at varying concentrations over a period of 112 days. The only protozoan observed throughout the run was the free-swimming ciliate, Colpoda. No stalked ciliates were found at all. In my research, a variety of ciliates, ameboids, and flagellates were seen with the ameboids beginning to dominate.

TABLE 17. Fresh Protozoa Populations*

Livinston A/S	Bichem	Hydrobac
Aspidisca	none	none
Colacium		
Didinium		
Dimastigamoeba		
Epistylis		
Lionotus		
Mayorella		
Oxytricha		
Paramecium		
Peranema		
Polychaos		
Valkampfia		
Vorticella		

*LLMO did not have any protozoa

TABLE 18. Phenol Acclimated Protozoa Populations

Livingston A/S	Bichem	Hydrobac
Colacium	none	none
Epistylis		
Lionotus		
Mayorella		
Paramecia		
Valkampfia		

TABLE 19. 2-Chlorophenol Acclimated Protozoa Populations

Livingston A/S	Bichem	Hydrobac
Aspidisca	none	none
Colacium		
Epistylis		
Mayorella		

In my research, during 2-chlorophenol exposure, individual stalked ciliates were seen with an occasional flagellate(Aspidisca) and the ameboids, Valkampfia and Mayorella predominating. Figure 10 depicts relative microorganism predominance when exposed to organic waste.

G. Description of Higher Organism(Table 20)

Activated sludge contains a number of multicellular organisms such as rotifer, roundworms, segmented worms, and small arachnids. A list has been compiled by Gainey and Lord(17). The most frequently found organisms found in activated sludge are listed in Table 23. My findings are listed in Table 20. Exposure to phenol and 2-chlorophenol may have decreased their numbers, but these organisms do survive the phenol insults.

No literature was found regarding actived sludge multicellular microorganisms exposed to phenol or 2-chlorophenol.

Bichem and Hydrobac, again, should not contain any of these higher organisms unless contaminated.

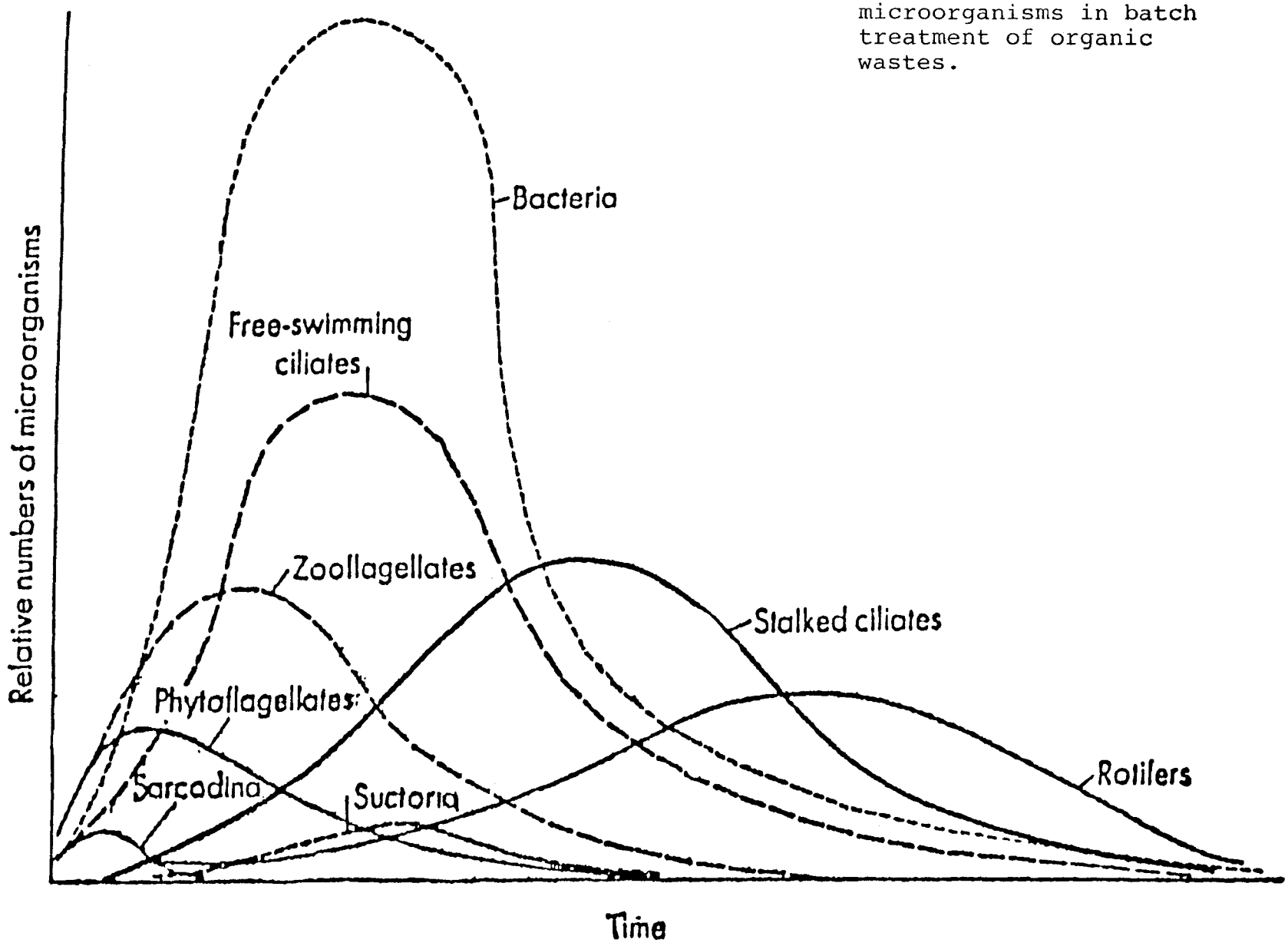


Figure 10. Relative predominance of microorganisms in batch treatment of organic wastes.

TABLE 20. Fresh Populations of Higher Organisms*

Livingston A/S	Bichem	Hydrobac
Philodina (rotifer)	none	none
Rhabiditoideae (nematode)		
Tyrelia (water mite)		

Phenol Acclimated Populations of Higher Organisms

Philodina (rotifer)	none	none
Rhabditoideae (nematode)		
Tyrelia (water mite)		

2-Chlorophenol Acclimated Populations of Higher Organisms

Philodina (rotifer)	none	none
Rhabitoideae (nematode)		
Tyrelia (water mite)		

Table 21. **Phenol degrading activity of fungi isolated from Lake Skadar.** (34)

Species	Strain	After 3 weeks of incubation in mineral-salts medium with phenol (500 ppm).		
		Growth	Spor.	%phenoldegr.
Alternaria alternata	SK16-21	++	+	4.82
Aspergillus flavipes	SK 7-13	+++	+	78.43
A. niger	SK 7-14	+++	+	41.57
A. protuberus	SK10- 6	+++	+	98.77
A. repens (Eurotium repens)	SK15- 6	+	-	0
A. repens (Eurotium repens)	SK16-19	++	±	8.11
A. versicolor	SK12- 3	+	-	0
A. versicolor	SK15- 3	+	-	0
A. versicolor	SK15- 7	++	+	3.95
Botryotrichum piluliferum	SK 2- 1	+++	+	45.69
B. piluliferum	SK 8- 1	++	+	19.21
Cladosporium herbarum	SK16- 3	++	+	99.01
C. sphaerospermum	SK 7- 7	+	-	0
C. sphaerospermum	SK16- 8	±	-	0
C. sphaerospermum	SK16-18	++	+	6.58
C. sphaerospermum	SK16-23	+++	+	59.10
C. sphaerospermum	SK17- 2	++	+	9.01
Penicillium brevi-compactum	SK16-17	+++	+	85.29
P. brevi-compactum	SK17- 6	+++	+	83.33
P. brevi-compactum	SK17-11	+++	+	2.94
P. chrysogenum	SK15- 1	+++	+	98.46
P. chrysogenum-notatum	SK 1-13	+++	+	1.96
P. chrysogenum-notatum	SK10- 4	+++	+	8.33
P. chrysogenum-notatum	SK10-10	±	-	0
P. chrysogenum-notatum	SK16-27	+++	+	3.92
P. chrysogenum-notatum	SK16-38	+++	+	10.59
P. cyclopium	SK10-11	+++	+	3.95
P. cyclopium	SK16- 4	+++	+	98.77
P. multicolor	SK16-32	+++	+	25.49
P. notatum	SK12- 9	+++	+	98.33

Table 21. continued

Species	Strain	After 3 weeks of incubation in mineral-salts medium with phenol (500 ppm).		
		Growth	Spor.	%phenoldegr.
P. notatum	SK15- 4	+++	+	98.92
P. notatum	SK16-13	+++	+	8.23
P. puberulum	SK15- 7	+++	+	3.95
P. stipitatum (Talaromyces stipitatus)	SK12- 4	±	-	0
P. wortmannii (Talaromyces wortmanii)	SK15- 5	+++	+	92.92
Phoma herbarum	SK12- 5	++	+	21.05
P. macrostoma var. incolorata	SK16-29	+++	+	15.69
Trichoderma viride	SK 7-12	+++	+	9.80
Trichothecium roseum	SK 7-19	+	+	1.96

Growth: +++ compact mycelial mass up to 4 mm thick; ++ mycelial mass very thin, not compact; + weak; - none.

Sporulation: + present; ± uncertain; - not seen

TABLE 22. The most frequently observed species of protozoa in activated sludge. (10)

Anthophysa vegetans

Oicomonas steinii

Oicomonas termo

Bodo caudatus

Bodo lens

Cercobodo carssicauda

Cercobodo longicauda

Pleuromonas jaculans

Amoeba actinophora

Amoeba guttula

Valkampfia limax

Carchesium polypinum

Opercularia sp.

Oxytricha fallax

Paramecium sp.

Vorticella alba

Vorticella campanula

Vorticella communis

Euglypha alveolata

Aspidisca costata

Aspidisca lynceus

Aspidisca robusta

Chilodonella cucullulus

Epistylis plicatilis

Euplotes caudiculatus

Litonotus fasciola

Vorticella convallaria

Vorticella microstoma

Vorticella nebulifera similis

Vorticella striata octava

Peranema trichophorum

Arcella vulgaris

Euglypha sp.

Carchesium polypinum

Euplotes moebiusi

Opercularia coarctata

Trachelophyllum pusillum

Vorticella fromenteli

Cochliopodium sp.

Chilodonella uncinata

Drepanomonas sp.

Hemiophrys sp.

TABLE 13 Frequency of Animal Microorganisms in Activated Sludge.(33)

	Protozoa	Rotifera	Crustacea
Occasionally found in large numbers.	Bursaria Chloromonas Raphidomonas		
Commonly found in small numbers	Actinophrys Amoeba Anthophysis Cercomonas Codonella Epistylis Monas Tintinnus Vorticella	Anuraea Conochilus Polyarthra Rotifera Synchaeta	Bosmina Cyclops Daphnia
Occasionally observed	Aineta Arcella Coleps Colpidium Cyphoderia Difflugia Enchelys Euglypha Euplotes Glaucoma Halteria Nassula Paramecium Pleuronema Raphidodendron Stentor Trichodina Uvella Zoothamnium	Asplanchna Colurus Eosphora Floscularia Lacinularia Mastigocerca Microcodon Monocera Monostyla Noteus Sacculus Triarthra	Alona Cypris Diaptomus Sida

CONCLUSIONS

Livingston activated sludge has a wide diversity of microorganisms. After exposure to ten days of 100 ppm phenol, followed by 10 more days of exposure to 20 ppm 2-chlorophenol, the Livingston activated sludge continued to exhibit a fairly diverse population.

After phenolic exposure, bacterial populations favored gram positive rods of the Bacillus genus and gram positive cocci belonging to Micrococcus.

The gram negative species, although the minority population, were largely of the genera Alcaligenes and Pseudomonas.

The dominant fungal genera surviving phenolic exposure were Candida, Trichosporon, and Penicillium.

The protozoa underwent a populational shift during exposure to phenols. Flagellate and ameboid numbers decreased during phenolic insults, while the ciliates continued to thrive.

Rotifers, round worms, and an occasional water mite also survived exposure to phenol and 2-chlorophenol.

Bichem, Hydrobac, and LLMO(the commercial populations)had limited variety in their species. During phenolic exposure, this diversity decreased such that only the gram positive Bacillus and the gram negative Pseudomonas remained in the Bichem and Hydrobac mixtures.

Any other microorganisms found in these artificial populations were probably contaminants.

This research has demonstrated that a heterogeneous population, such as that found in a municipal activated sludge, can biodegrade hazardous chemicals and do it more effectively than a more homogeneous population.

APPENDIX A

Detailed Procedures

STREAKING

1. Holding the agar plate with one hand, sterilize the loop and pick up a small amount of the colony and streak, back and forth, the top quarter of the plate as shown in Figure 11a.

2. Starting at the top of the plate, draw down vertically, Figure 11b.

3. Streak back and forth and in a downward direction starting halfway down the plate, Figure 11c.

4. Turn the plate 45 and repeat step 3.(Figure 11)d.

5. Turn the plate 90 and repeat step 3.(Figure 11)e.

FLAGELLA STAIN(Walc-Pokrzywnicki)

Fixative:	Ethanol	6 parts
	Chloroform	3 parts
	Formalin	1 part
Mordant:	20% tannic acid	3 parts
	5% ferric chloride	1 part

Silver stain: Dissolve a few grams of silver nitrate in approximately 100 mls distilled water. Set approximately 10 ml aside in a test tube. Add concentrated ammonium hydroxide solution drop by drop until the heavy precipitate formed at the beginning just dissolves, leaving a faint opalescence. If too much ammonia is added, add a little reserve silver nitrate

solution. The final solution should be clear by transmitted light but faintly opalescent against a black background. Leave for 10 min. then correct if necessary. This solution keeps indefinitely.

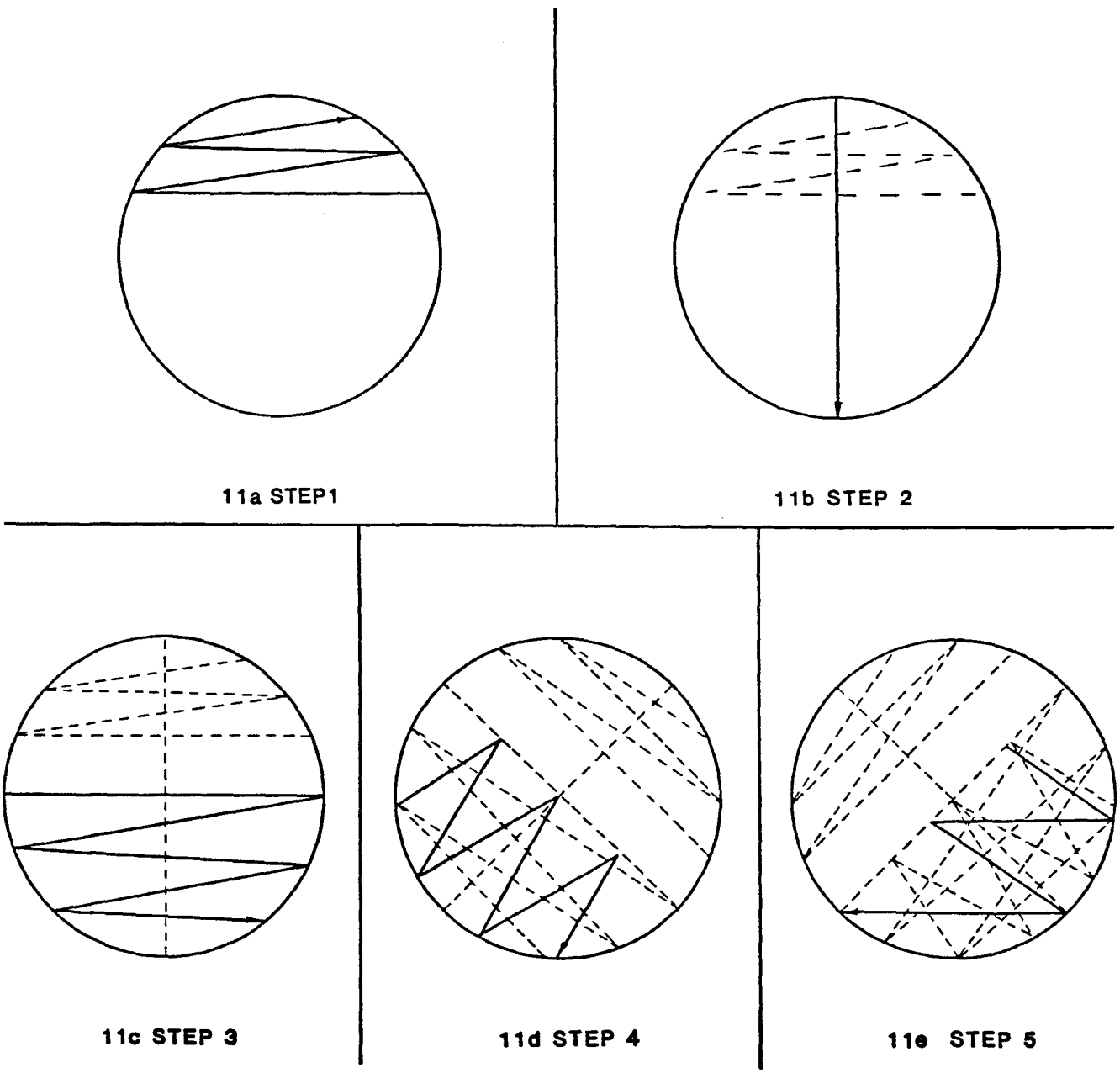
Organisms were grown in brain heart infusion broth. A loopful of broth was placed on a glass slide with a loopful of formalin and allowed to air dry.

Place in fixative for three minutes, wash with distilled water and drain. Flood with mordant for 5-6 minutes. Wash with distilled water, drain, and dry the back of the slide.

Pour silver stain over the wet film through Whatman #1 filter paper and leave 15-20 min(I left it on approximately 45 min.). Wash with water and examine. The organisms are stained black and the flagella are brown.

STREAKING OF AGAR PLATES

FIGURES 11a-e



11a STEP1

11b STEP 2

11c STEP 3

11d STEP 4

11e STEP 5

FLAGELLA STAIN(Leifson method)

Solution 1:	NaCl	1.5g
	Distilled water	100ml
Solution 2:	Tannic acid	3.0g
	Distilled water	100ml
Solution 3:	Pararosaniline acetate	0.9g
	Pararosaniline HCl	0.3g
	Ethanol, 95%	100ml

Mix equal volumes of solution 1 and 2 then add two volumes of this to one volume of solution 3. Drop onto the slide and allow to remain until the liquied form a golden sheen. Rinse with distilled water. Flagella stain red.

CATALASE TEST(for Gram positive bacteria)

Colonies were grown on plate count agar for 24 hours then a few drops of 3% hydrogen peroxide were sprinkled on the colonies. Positive results were indicated by bubbles.

SLIDE COAGULASE TEST

One or two colonies of gram positive organisms are swirled in a drop of water on a glass slide. If no clumping occurs in 10-20 seconds, dip a straight wire into human or rabbit plasma and stir the bacterial suspension in it. Clumping should occur.

PHOSPHATASE TEST

Add one milliliter of 1% Seitz-filtered phenolphthalein phosphate to 100 ml melted nutrient agar and pour into plates. Inoculate and incubate overnight. Expose to ammonia vapor. Colonies of phosphatase-positive staphylococci will turn pink due to free phenolphthalein. Staphylococcus aureus is positive. Coagulase negative staphylococci and micrococci are usually phosphatase negative.

OXIDASE TEST

Add 0.01g N,N,N',N'-tetramethyl-p-phenylenediamine to 1 ml of distilled water. In a petri dish cover, place Whatman #1 filter paper and soak with the phenylenediamine mixture. Streak colonies on paper and check for purple color within 10-15 seconds.

COUNTING ORGANISMS

1) Add a few drops of formalin to the 50 microliters of solution that was pipetted into the counting chamber.

2) A glass coverslip was placed over the chamber and then the organisms observed under 100x.

3) The numbers were then averaged per square and multiplied by 2x10 times the dilution factor. This number was the average amount of organisms per cubic centimeter.

BACTERIAL POPULATION PREPARATIONS

BI-CHEM and HYDROBAC

12.5 g of flakes were added to one liter of distilled water and agitated for two hours at room temperature. A 600 ml aliquot was decanted into the lucite reactor. The reactor volume was brought up to 1000ml with aerated, distilled water. Phenol feeding solution was then added.

LLMO

250 ml of well-shaken liquid was added to a reactor and the volume was brought up to one liter. The mixture was allowed to aerate for 24 hours to allow for the hydrogen sulfide to strip out.

APPENDIX B

Growth Media Formulations

Brain Heart Infusion

Prepared by Difco

Cellulose Decomposing Bacteria

$(NH_4)_2SO_4$	0.1g
K_2HPO_4	0.1g
$MgSO_4*7H_2O$	0.05g
$CaCO_3$	0.2g
NaCl	trace
Agar	2.0g
Water to	100ml

Autoclave then add a strip of filter paper.

Cetrimide Agar

gelysate pancreatic	
digest of gelatin	2.0g
$MgCl_2$	0.14g
K_2SO_4	1.0g
Agar	1.4g
Cetrimide	0.03g
Water to	100ml

Autoclave.

Cornmeal agar or Czapek agar w/rose bengal

As per instruction(Difco)then add 33mg rose bengal per 100ml solution.

Dextrose Tryptone Agar

Tryptone	1.0g
Dextrose	0.5g
Agar	1.5g
Bromcresol purple	0.004g
Water to	100ml

Autoclave.

Floc-forming bacteria

Protease-peptone	0.2g
Yeast extract	0.1g
Agar	2.0g
Water to	100ml

Autoclave

Motility Agar

Beef extract	0.3g
Peptone	1.0g
NaCl	0.5g
Agar	0.4g
Water to	100ml

Autoclave

Nitrifying Bacteria

$(NH_4)_2SO_4$	0.005M
NaCl	0.005M
KH_2PO_4	0.001M
$MgSO_4*7H_2O$	0.001M
$CaCO_3$	1.0g
Agar	2.0g
Water to	100ml

Autoclave then incubate in the dark at 25 C for 1-2 weeks.

OXA

Peptone	10.0g
Glucose	10.0g
Oxgall(bovine bile)	15.0g
Agar	20.0g
Water to	1000ml

Add 50mg streptomycin and 10mg penicillin G after autoclaving.

Filter sterilize antibiotics.

Phenol-utilizing Bacteria

H_2KPO_4	0.1g
$MgSO_4*7H_2O$	0.02g
NaCl	0.01g
$CaCl_2$	0.01g
$FeCl_3$	0.002g
$(NH_4)_2SO_4$	0.1g
Phenol	0.07M(or concentration desired)
Agar	2.0g
Water to	100ml

Autoclave

Sabouraud

Peptone	1.0g
Glucose	4.0g
Agar	1.5g
Water to	100ml

Autoclave.

Thiobacillus

$Na_2S_2O_3*5H_2O$	1.0g
NH_4Cl	0.1g
KH_2PO_4	0.1g
$MgCl_2*6H_2O$	0.05g
Water to	100ml
Agar	2.0g

Autoclave then inoculate and incubate in the dark at $30^{\circ}C$ for 1-2 weeks.

YM Agar

Yeast extract	0.3g
Malt extract	0.3g
Peptone	0.5g
Glucose	1.0g
Agar	2.0g
Water to	100ml

After autoclaving, cool to approximately 50°C and add 0.7ml of 1N HCl.

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