A DETERMINATION OF THE BACTERIAL AND FUNGAL FLORA OF NORMAL AND IRON CONTAINING FISH AQUARIA

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INTRODUCTION

Roeder and Roeder (1963) observed a noticeable increase in the growth of the swordtail, Xiphophorus helleri, and the hybrid of the swordtail and platyfish, Xiphophorus maculatus, when small amounts of ferrous salt were added to aquaria. They also observed that the ferrous sulfate was quickly oxidized to ferric hydroxide and precipitated to the bottom of the tanks. They continued the experiment by studying four aquaria containing the hybrid fishes and four containing swordtails. To the first tank of each group they added no ferrous sulfate; to the second, 25 mg; to the third, 50 mg; and to the fourth, 100 mg of ferrous sulfate. Their results showed not only an increase in fish growth in each of the tanks containing iron, but also a growth increase that was proportional to the amount of iron added.

I began experiments to determine whether the growth increase in the swordtails was related to a variation in the microflora of the fish tanks caused by the daily addition of iron. The experiments consisted of a comparison of predominant bacteria in fish tanks to which no iron had been added and tanks to which varying concentrations of iron had been added over a period of thirteen months.

Very little research has been done in the field of fresh water bacteriology except for studies of coliform bacteria and other water contaminants. Pelczar and Reid (1958) list several genera which normally occur in natural waters. These are Pseudomonas, Flavobacterium, Aerobacter, Bacterium, Hyphomicrobiales, Azobacter, Beggiatoa, Brevibacterium, Micromonospora, Paracolobactrum, Spirochaeta, Thiobacillus, and Vibrio. Prescott and Winslow (1946) describe water

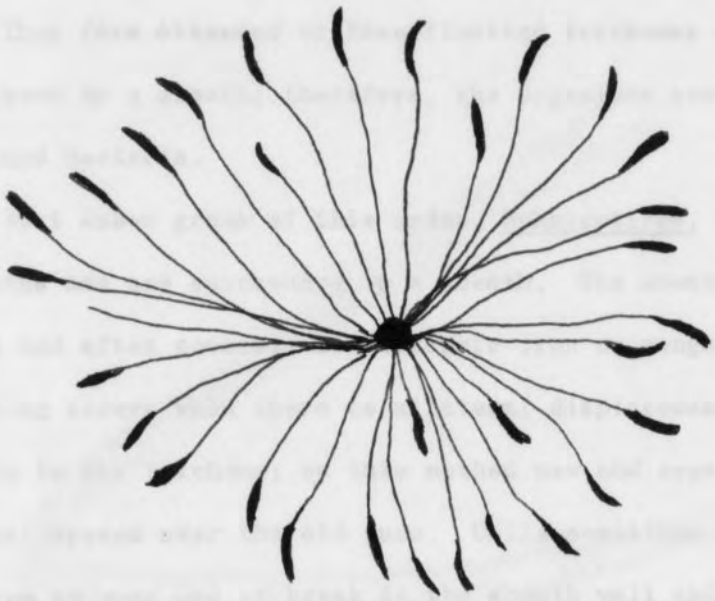
bacteria as belonging to the following groups: (a) fluorescent, (b) violet, red, or yellow chromogenic, (c) coliform types, (d) non-gas forming rods which do not produce fast spreading colonies and may or may not acidify milk and liquefy gelatin, (e) the spore forming Bacillus subtilis, and (f) white, yellow, and pink cocci.

Very limited studies have been made of water bacteria that are known to oxidize ferrous salts to ferric hydroxide. Breed, Murray, and Smith (1957) place most of these organisms in the orders Pseudomonadales or Chlamydobacteriales. The family Caulobacteraceae of Pseudomonadales consists of non-filamentous, rod-shaped bacteria that produce a fine stalk or holdfast by which they attach to some object. These organisms normally form stalks by oxidizing soluble ferrous salts to ferric hydroxide, which is secreted in thin layers from one side of the cells. These bacteria are gram negative and non-motile in the attached form. They multiply by transverse fission. The daughter cells either remain attached or swim away by means of polar flagella to form new colonies.

There are two genera in this family, Caulobacter and Gallionella. In the genus Caulobacter, the long axis of the cell coincides with the axis of the stalk. Groups of these cells are often found attached to a common holdfast and form a rosette pattern. A diagram of Caulobacter cells is found in Figure I, (page 3). Organisms of the Gallionella genus are curved or kidney-shaped with the long axis of the cell perpendicular to the long axis of the stalk. These stalks are long and often twisted. Gallionella cells are illustrated in Figure II, (page 3).

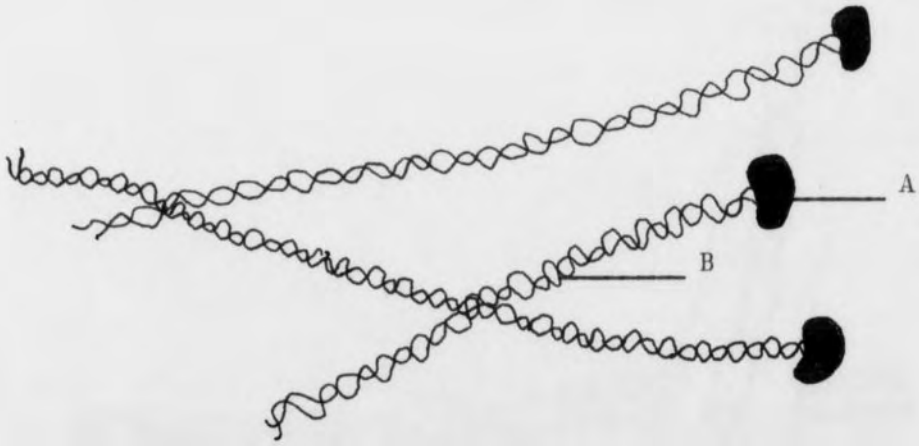
Pelczar and Reid (1958) describe the bacteria of the order

FIGURE I



Drawing of Caulobacter cells attached to a common holdfast and exhibiting a rosette pattern. Caulobacter range from 0.5 to 1.2 microns in width and from 1.5 to 3.0 microns in length.

FIGURE II



Drawing of Gallionella. (A) The bacterial cell. (B) Colloidal ferric hydroxide deposited from the concave side of the cell forms flat bands or ribbons which extend from the cell. The individual ribbons twist and may become entangled with other ribbons. (Both drawing from M. J. Pelczar and R. D. Reid, Microbiology, McGraw-Hill Book Company, Inc., New York, 1958, p. 126.)

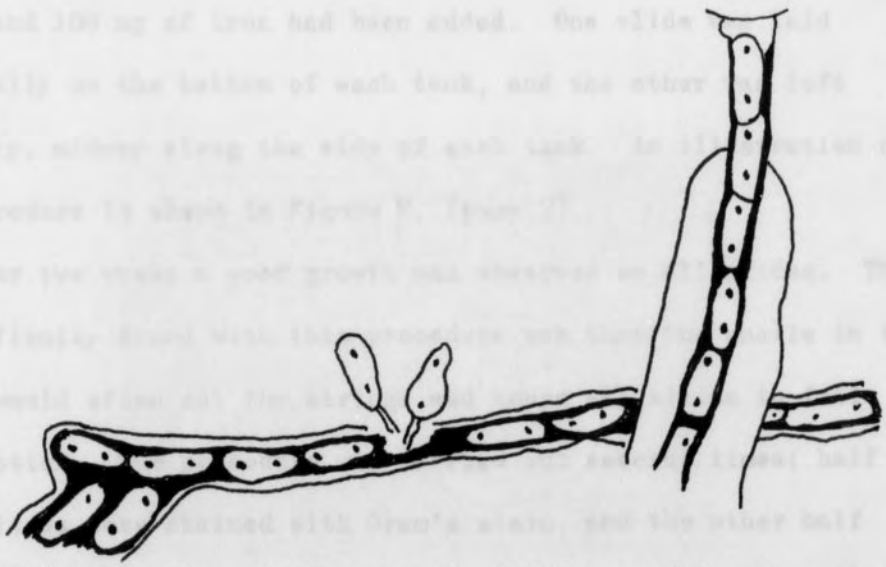
Chlamydobacteriales as being colorless, gram negative, algal-like organisms. They form attached or free-floating trichomes which are usually enclosed by a sheath; therefore, the organisms are commonly called sheathed bacteria.

In the best known genus of this order, Sphaerotilus, the cells occur in chains and are surrounded by a sheath. The sheath is secreted by the cells and often consists of insoluble iron or manganese oxides. False branching occurs when there is a lateral displacement of several cells in the trichome; by this method new and separate trichomes are imposed over the old ones. Cells sometimes leave the trichomes from an open end or break in the sheath wall and move by means of polar flagella to form new colonies. These bacteria are illustrated in Figures III and IV, (page 5).

FIGURE III

Drawing of sheath and cells of Sphaerotilus natans from microscopic slide stained with nigrosin. Dimensions of individual cells are 1 by 2 to 6 microns and the sheaths may reach a length of several millimeters.

FIGURE IV



Drawing of Sphaerotilus showing sheath, holfast, motile swarmers, and false branching. (Both diagrams from M. J. Pelczar and R. D. Reid, Microbiology, McGraw-Hill Book Company, Inc., New York, 1958, p. 127.)

MATERIALS AND METHODS

The first experiments carried out in this project were devised to detect organisms in either the family Caulobacteraceae or order Chlamydobacteriales. Henrici (1933) has demonstrated the most successful method for isolating these organisms. He observed that the attached forms of the iron and sheathed bacteria could be cultured by submerging clean glass microslides in water for two weeks. Iron bacteria present in the water would become attached to the slides and could be observed microscopically after the slides had been properly stained.

A long string, tied to an identification card, was attached with adhesive tape to a thoroughly cleaned slide. Adhesive tape was used because it is relatively inert and would not change the environment of the tanks. Two slides were placed in each of the four types of aquaria: those to which no iron had been added, and those to which 25, 50, and 100 mg of iron had been added. One slide was laid horizontally on the bottom of each tank, and the other was left vertically, midway along the side of each tank. An illustration of this procedure is shown in Figure V, (page 7).

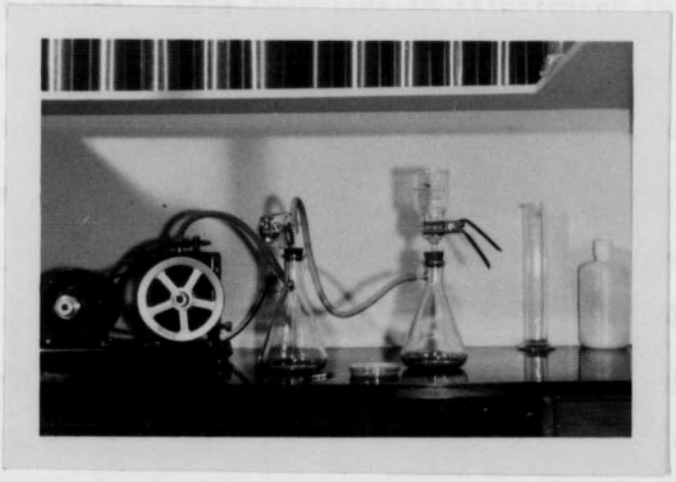
After two weeks a good growth was observed on all slides. The only difficulty found with this procedure was that the snails in the aquaria would often cut the strings and cause all slides to fall to the bottom. The procedure was carried out several times; half of the slides were stained with Gram's stain, and the other half were stained with nigrosin. No typical iron bacteria were observed on the slides.

The next experiments were designed to culture on artificial

FIGURE V

The Henrici method of isolating iron bacteria. Microslides are placed in the tanks for two weeks and later observed microscopically.

FIGURE VI



The Millipore apparatus for concentrating water samples.

media any iron bacteria which might be present in the aquaria. Problems then arose as to which types of media to use. Little study has been made of the iron bacteria, principally because they are so difficult to culture. Even though no practical culture media were listed in the literature, other media, devised in early attempts to isolate iron bacteria, proved to be of historical significance. Levine and Schoenlein (1930) described one medium made of pea infusion; another called for a sterile brick; and still another required water specifically from the Moldau River.

Two preparations, (1) nutrient agar, and (2) nutrient agar containing 1 gram of ferrous sulfate per liter of solution, were chosen for this second attempt to isolate iron bacteria. The Millipore filter with a pore size of .45 microns was used to concentrate the number of microflora in each sample. The Millipore apparatus is shown in Figure VI, (page 7). Two 100 ml samples were obtained from each of the four aquaria. The tank water was first stirred. Then a propipette was used to suction water from the bottom of each aquarium into sterile pipettes. This technique is illustrated in Figure VII, (page 9). After each sample was run through the sterile Millipore filter, the filter was rubbed across either nutrient agar or iron agar. The first plates were incubated in the room where the tanks were housed. This allowed the bacteria to grow at approximately the temperature of the tanks. However, growth appeared on these plates only after four to five days of incubation. The next plates were incubated at 37°C to avoid any contamination that might occur during a lengthy incubation time. No organisms resembling iron bacteria were isolated.

FIGURE VII

The method of obtaining samples from the fish tanks using a propipette and a sterile pipette.

FIGURE VIII

Quebeck colony counter.

A third attempt was made to detect iron bacteria in the fish aquaria. Random samples were taken from the tanks and observed unstained in a wet mount, as well as stained with Gram's technique and nigrosin. No organisms resembling iron bacteria were observed, and no further attempts were made to isolate this particular group of bacteria.

The next experiments consisted of colony counts on the tank water to determine the bacterial concentration in each of the four aquaria. Standard public health methods for examination of water were used. (Standard Method for Examination of Water, Sewage, and Industrial Wastes, 1936, eighth edition.) Before each sample was obtained, the tank water was stirred. 10 ml samples were then pipetted from the middle of each tank. Nutrient agar pour plates were set up using undiluted samples and dilutions of 1 part tank water to 10, 100, 1000, 10,000, and 100,000 parts sterile distilled water. The dilutions from each of the three tanks containing iron were also plated in nutrient agar to which iron had been added in the same concentration as in the tank from which the sample was taken. Counts were made after 24 and 48 hours of incubation at 37°C using the Quebeck colony counter shown in Figure VIII, (page 9).

The bacteria and fungi cultured from the four tanks on nutrient and iron agar were isolated by repeated plating of each different colony until it appeared pure in terms of colonial and microscopic characteristics. An attempt was made to identify the bacteria isolated on nutrient agar by running standard biochemical tests on each organism. The same tests were run on bacteria isolated on iron agar from the tank containing 100 mg of iron. The one fungal culture

present on all plates was studied colonially on Sabouraud's medium and morphologically from a potato dextrose culture stained with lactophenol cotton blue.

A quantitative determination of the dissolved oxygen content of the aquaria by the Winkler method was also made. All results are recorded in tables in the next section.

RESULTS

No organisms on the hanging slides, on the nutrient and iron agar plates, or on the moist and stained preparations resembled morphologically the iron or sheathed bacteria. More growth was observed on hanging slides left at the bottom of tanks than on those hanging vertically along the side of tanks. On the first iron plates it was apparent that 1 gram of ferrous sulfate per liter of solution had inhibited all bacterial growth, for only the fungus was isolated on this medium.

Results of the colony counts can be found in Tables I and II, (pages 13-14). Total counts on both iron and nutrient agar from the tanks containing no iron, 25 mg iron, and 50 mg iron were between 3×10^5 and 7×10^5 bacteria per milliliter of tank water. In the tank containing 100 mg of iron the total colony counts on both iron agar and nutrient agar were ten times greater than in the other three tanks; on nutrient agar 5.5×10^6 colonies per milliliter were counted, and on iron agar 4.8×10^6 colonies per milliliter of tank water were counted. There was no striking difference between the colony counts from any of the tanks on nutrient agar as opposed to the counts on iron agar.

The colonial and microscopic descriptions of bacteria isolated from each of the four aquaria are recorded in Table III, (page 15). The same eight colony types were present in all four aquaria. All bacteria were gram negative rods (Figure IX, page 16) except colony 4, which was a gram positive, non-spore forming rod (Figure X, page 16).

Results of the biochemical tests are recorded in Tables IV and

TABLE I

COLONY COUNTS OF BACTERIA FROM AQUARIA WITH 0 and 25 mg OF IRON MADE FROM COLONIES ISOLATED ON NUTRIENT AND IRON AGAR

TNC - too numerous to count.

The number of colonies on a plate multiplied by the sample dilution equals the number of bacteria per milliliter of sample.

SAMPLE DILUTION	COLONIES ON NUTRIENT AGAR		COLONIES ON NUTRIENT IRON AGAR	
0 mg iron	24 hours	48 hours	24 hours	48 hours
undiluted	TNC	TNC		
10	TNC	TNC		
10^2	TNC	TNC		
10^3	394	529		
10^4	52	57		
10^5	9	5		
25 mg iron				
undiluted	TNC	TNC	TNC	TNC
10	TNC	TNC	TNC	TNC
10^2	TNC	TNC	TNC	TNC
10^3	341	539	370	523
10^4	31	50	49	50
10^5	7	8	2	5

TABLE II

COLONY COUNTS OF BACTERIA FROM AQUARIA WITH 50 and 100 mg OF IRON MADE FROM COLONIES ISOLATED ON NUTRIENT AND IRON AGAR

TNC - too numerous to count.

The number of colonies on a plate multiplied by the sample dilution equals the number of bacteria per milliliter of sample.

SAMPLE DILUTION	COLONIES ON NUTRIENT AGAR		COLONIES ON NUTRIENT IRON AGAR	
0 mg iron	24 hours	48 hours	24 hours	48 hours
undiluted	TNC	TNC	TNC	TNC
10	TNC	TNC	TNC	TNC
10^2	TNC	TNC	TNC	TNC
10^3	224	362	270	431
10^4	22	32	47	36
10^5	4	4	2	4
25 mg iron				
undiluted	TNC	TNC	TNC	TNC
10	TNC	TNC	TNC	TNC
10^2	TNC	TNC	TNC	TNC
10^3	TNC	TNC	TNC	TNC
10^4	181	257	184	269
10^5	43	67	38	58

TABLE III

MICROSCOPIC AND COLONIAL DESCRIPTION OF THE EIGHT COLONIES ISOLATED FROM EACH OF THE FOUR AQUARIA

ORGANISMS ABCDE*	MICROSCOPIC DESCRIPTION	COLONIAL CHARACTERISTICS
3	small gram negative rods	small, circular, mucoid, bluish white, fast spreading colonies
4	small gram positive rods	hazy, circular, fast spreading colonies
6	fungus with sepate hyphae, chlamydospores, and oval spores held together in a jelly-like mass on aerial hyphae.	fungus with white, fine, hair-like mycelium forming a circular colony and later developing into a red cushion-like structure.
7	small gram negative rods, sometimes in short chains	small, circular, mucoid, fast spreading, pinkish-white colonies
9	long, thin gram negative rod	almost clear, pin point colonies
11	small gram negative rods, sometimes found in short chains	mucoid, dark yellow colonies with round entire margins
12	small gram negative rods, sometimes found in short chains	medium, circular, white colonies developing into clover shaped, hard colonies
16	small gram negative rods	medium, fat, circular, mucoid, yellowish white colonies

*ABCDE

A- Colonies isolated on nutrient agar from the tank with 0 mg of iron.

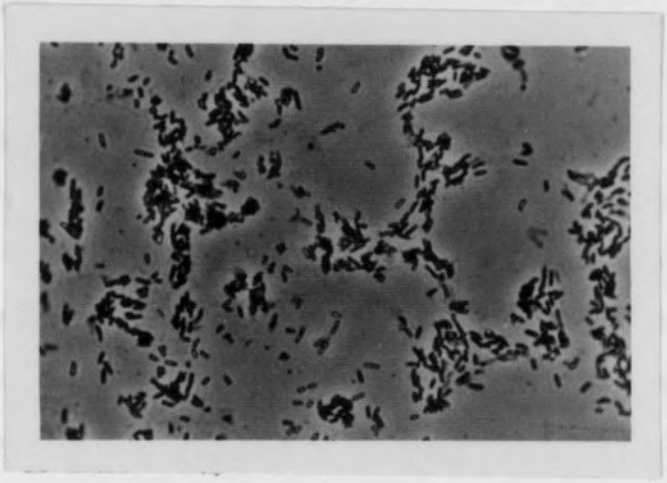
B- Colonies isolated on nutrient agar from the tank with 25 mg of iron.

C- Colonies isolated on nutrient agar from the tank with 50 mg of iron.

D- Colonies isolated on nutrient agar from the tank with 100 mg of iron.

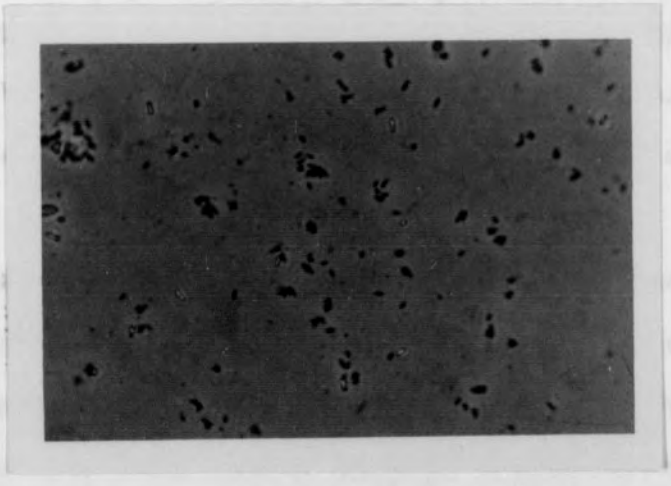
E- Colonies isolated on iron agar from the tank with 100 mg of iron.

FIGURE IX



Typical gram negative rods isolated from the fish tanks. This photograph was made of a slide under the phase contrast microscope with 400 total magnification.

FIGURE X



The gram positive rod isolated from the fish tanks. This photograph was made of a slide under the phase contrast microscope with 400 total magnification.

V, (pages 18-19). Colonies designated 4, 7, 11, and 12 from the aquarium without iron gave the same biochemical reactions as colonies 4, 7, 11, and 12 isolated from the aquaria containing varying concentrations of iron. The addition of iron had evidently not affected these bacteria biochemically. Colonies 3, 9, and 16 isolated from the aquarium to which no iron was added appeared identical in colonial and microscopic characteristics to colonies 3, 9, and 16 isolated from the aquaria containing varying concentrations of iron; however, they differed in biochemical reactions. Colonies 3, 9, and 16 from the aquaria with iron oxidized several sugars to an acid end-point. Colonies 3, 9, and 16 from the aquarium to which no iron was added failed to produce an acid reaction in carbohydrate media. In fact, none of the organisms isolated from the aquarium without iron were able to oxidize sugars. An additional biochemical test revealed that the sugar oxidation carried out by colonies 3, 9, and 16 from the iron aquaria was a fermentative process rather than aerobic oxidation.

Every organism isolated reduced nitrates to nitrites. All bacteria except those from colony 11 grew in citrate broth; consequently, they were probably autotrophic. None of the bacteria oxidized lactose; thus, no typical coliform bacteria were isolated. Most of the bacteria were motile.

All of these bacteria were isolated on nutrient agar plates from the last colony count. As a final check on the biochemical results, a study was made of the bacteria isolated on iron agar from the tank containing 100 mg of iron. These organisms seemed identical in colonial, microscopic, and biochemical characteristics to the other bacteria isolated from the iron tanks on nutrient agar.

TABLE IV

RESULTS FROM THE BIOCHEMICAL TESTS RUN ON THE SAME SEVEN BACTERIAL COLONIES - 3, 4, 7, 9, 11, 12, 16 - ISOLATED FROM THE AQUARIUM TO WHICH NO IRON HAD BEEN ADDED

A- Refers to the same colony groups as explained in the key to **Table** III.

ORGANISM	A-3	A-4	A-7	A-9	A-11	A-12	A-16
gelatin	+ 5 days	+	-	-	-	-	+
citrate	+	+	+	+	-	+	+
H_2S	-	+ 4 days	-	-	-	-	+ 4 days
motility	+	+	+	-	+	-	+
nitrate	+	+	+	+	+	+	+
litmus milk	-	pepton-ization	fermen-tation	-	-	-	pepton-ization
methyl red	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-	-	-
arabinose	-	-	-	-	-	-	-
fructose	-	-	-	-	-	-	-
starch	-	-	-	-	-	-	-
galactose	-	-	-	-	-	-	-
mannose	-	-	-	-	-	-	-
xylose	-	-	-	-	-	-	-
sucrose	-	-	-	-	-	-	-
lactose	-	-	-	-	-	-	-
maltose	-	-	-	-	-	-	-
mannitol	-	-	-	-	-	-	-
dextrose	-	-	-	-	-	-	-
sugar base control	-	-	-	-	-	-	-

TABLE V

RESULTS FROM THE BIOCHEMICAL TESTS RUN ON THE SAME SEVEN BACTERIAL COLONIES - 3, 4, 7, 9, 11, 12, and 16 - ISOLATED FROM THE AQUARIA TO WHICH IRON HAD BEEN ADDED

BCDE- Refer to the same colony groups as explained in the key to Table III.

ORGANISMS	BCDE 3	BCDE 4	BCDE 7	BCDE 9	BCDE 11	BCDE 12	BCDE 16
gelatin	+	+	-	-	-	-	+
citrate	+	+	+	+	-	+	+
H_2S	-	+ 4 days	-	-	-	-	-
motility	+	+	+	-	+	-	+
nitrate	+	+	+	+	+	+	+
litmus milk	peptonization	peptonization	fermentation	-	-	-	peptonization
methyl red	+	-	-	-	-	-	+
Voges-Proskauer	-	-	-	-	-	-	-
arabinose	(A)	-	-	weakly A	-	-	(A)
fructose	(A)	-	-	-	-	-	(A)
starch	(A)	-	-	-	-	-	(A)
galactose	(A)	-	-	-	-	-	(A)
mannose	(A)	-	-	-	-	-	(A)
xylose	-	-	-	weakly A	-	-	-
sucrose	(A)	-	-	-	-	-	(A)
lactose	-	-	-	-	-	-	-
maltose	(A)	-	-	weakly A	-	-	(A)
mannitol	(A)	-	-	-	-	-	(A)
dextrose	(A)	-	-	weakly A	-	-	(A)
sugar base control	-	-	-	-	-	-	-

Identification of the bacteria isolated from the fish aquaria was made on the basis of characteristics most closely resembling a particular genus or species described in Bergey's Manual. The key to the identification is noted in Table VI, (page 21-22). All of the bacteria were identified as common inhabitants of fresh water.

The microscopic and colonial characteristics of the fungus isolated from all the aquaria are found in Table III, (page 15). Figure XI, (page 23) shows the colonial characteristics of this fungus, and Figures XII-XIV, (page 24) show the microscopic characteristics. The fungus first became noticeable because it was the only organism that would grow on nutrient agar to which 1 gram of ferrous sulfate was added. Since the fungus also grew on nutrient agar without iron, it was concluded that the iron agar had only inhibited bacterial growth and consequently allowed the slower growing fungus to proliferate. From the colony count plates the fungus was found in the same concentration in the aquarium without iron as in the aquaria containing iron. The fungus did not appear to interfere with the growth of the bacteria, for the bacteria grew well in the presence of the fungus. Some bacteria were even growing between the fungal hyphae.

The fungus is identified as a Fusarium, a member of the class Deuteromycetes (Table VI, page 21). It is characterized by its production of a cushion shaped sporodochium which develops after the white mycelium has differentiated. The sporodochium is whitish to pink when the fungus is young. As the fungus grows, the sporodochium enlarges and produces pigments which give the colony an orange pink to wine red color. These pigments diffuse throughout the medium as the sporodochium becomes older. The sporodochium is almost mushroom

TABLE VI

IDENTIFICATION OF BACTERIAL AND FUNGAL COLONIES ISOLATED FROM THE FOUR AQUARIA

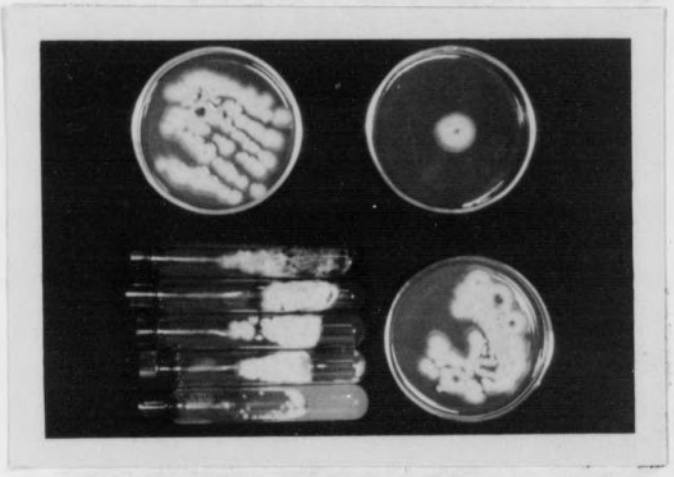
ABCDE refer to the same colony groups as explained in the key to Table III.

The page numbers refer to the page in Bergey's Manual of Determinative Bacteriology, seventh edition, where the bacteria are described. The page reference for colony ABCDE--4, however, is found in the sixth edition of the manual.

COLONIES	IDENTIFICATION		
A------3	order:	Eubacteriales	
	family:	Achromobacteraceae	
	genus:	Achromobacter	
	species:	thalassius	p. 303.
BCDE---3	order:	Eubacteriales	
	family:	Achromobacteraceae	
	genus:	Flavobacterium	
	species:	dormitator (non-chromogenic strain)	p. 314.
ABCDE--4	order:	Eubacteriales	
	family:	Bacteriaceae	
	genus:	Achromobacter	
	species:	sulfureum	p. 609.
ABCDE--6 (fungus)	phylum:	Eumycophyta	
	class:	Deuteromycetes	
	family:	Tuberculariaceae	
	genus:	Fusarium	
ABCDE--7	order:	Eubacteriales	
	family:	Achromobacteraceae	
	genus:	Achromobacter	
	species:	superficialis	p. 306.
A------9	order:	Eubacteriales	
	family:	Achromobacteraceae	
	genus:	Achromobacter	
	species:	parvulus	p. 309.
BCDE---9	order:	Eubacteriales	
	family:	Achromobacteraceae	
	genus:	Achromobacter	
	species:	eurydice	p. 308.

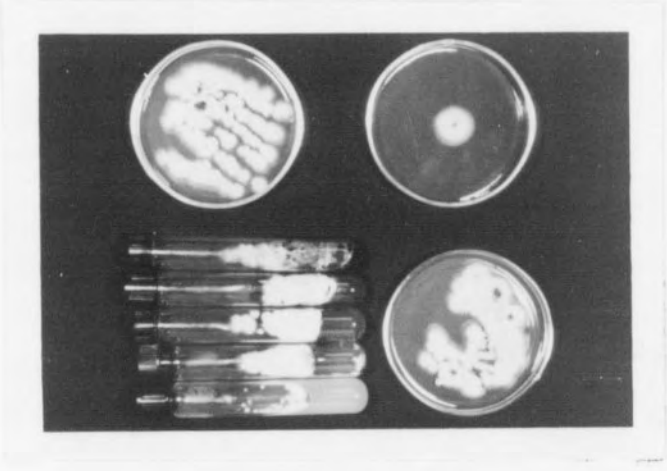
COLONIES	IDENTIFICATION		
ABCDE--11	order:	Eubacteriales	
	family:	Achromobacteraceae	
	genus:	Achromobacter	
	species:	pestifer	p. 306.
ABCDE--12	order:	Pseudomonadales	
	family:	Pseudomonadaceae	
	genus:	Pseudomonas	
	species:	stutzeri	p. 115.
A------16	order:	Eubacteriales	
	family:	Achromobacteraceae	
	genus:	Flavobacterium	
	species:	fucatum	p. 312.
BCDE---16	order:	Psuedomonadales	
	family:	Pseudomonadaceae	
	genus:	Pseudomonas	
	species:	xanthe	p. 180.

FIGURE XI



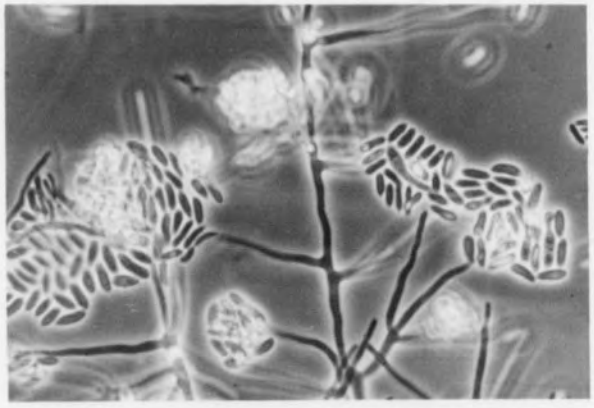
Colonial characteristics of the fungus isolated from all the fish tanks. These cultures were made on Sabouraud's medium. The outstanding characteristic of this fungus is the red sporodochium which develops in the center of the fungus colony after the white mycelium has differentiated.

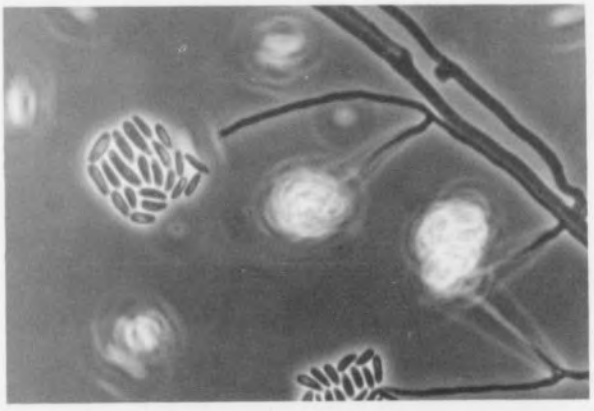
FIGURE XI

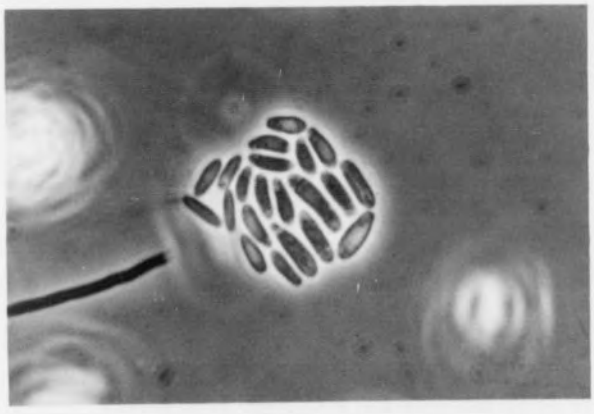


Colonial characteristics of the fungus isolated from all the fish tanks. These cultures were made on Sabouraud's medium. The outstanding characteristic of this fungus is the red sporodochium which develops in the center of the fungus colony after the white mycelium has differentiated.

FIGURES XII - XIV





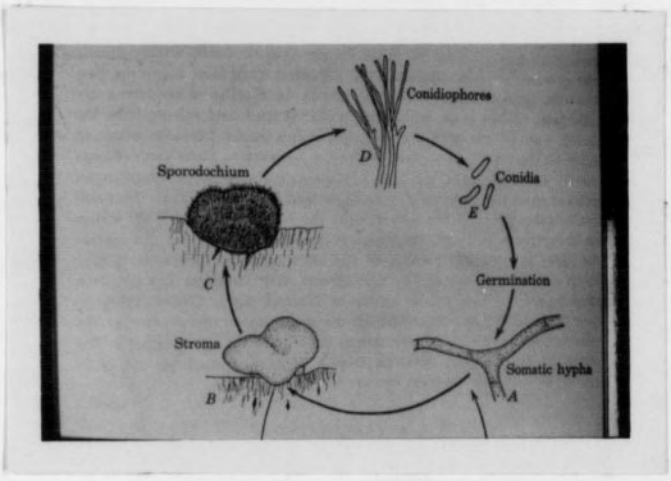


Microscopic views of the fungus isolated from the fish tanks showing masses of oval conidia attached to thin conidiophores. These photographs were made from slide cultures mounted in lactophenol cotton blue under the phase contrast microscope with 256 total magnification.

shaped at maturity. Its surface consists of thin conidiophores on the tips of which are borne several oval, multiseptate conidia, held together in a spherical, gelatinous mass. The conidia are very easily dispersed from this mass. Chlamydospores, or thickenings of the hyphal cells in the mycelium, are other asexual spores characteristic of this fungus. Figure XV, (page 26) shows the life cycle of the Fusarium.

The results of the Winkler determination of the dissolved oxygen content of the aquaria revealed a decrease in the amount of oxygen on the addition of iron to the aquaria. Four of these determinations were made, and all yielded similar results. The results of the fourth determination are found in Table VII, (page 27).

FIGURE XV



Life cycle of the fungus Fusarium. This diagram was taken from Constantine John Alexopoulos, Introductory Mycology, John Wiley & Sons, Inc., New York, p. 267.

TABLE VII

RESULTS OF THE WINKLER DETERMINATION
OF THE DISSOLVED O_2 CONTENT OF THE WATER FROM THE AQUARIA

IRON CONTENT OF TANK	DISSOLVED O_2 p.p.m.	AVERAGE p.p.m.
0 mg iron	1.80	2.00
0 mg iron	2.20	
25 mg iron	1.12	1.14
25 mg iron	1.16	
50 mg iron	1.00	1.00
50 mg iron	1.00	
50 mg iron	1.00	
50 mg iron	1.00	

DISCUSSION AND CONCLUSIONS

It can not be conclusively stated that no iron or sheathed bacteria are present in these fish aquaria. But using the various accepted methods for detecting the organisms, as well as a few original procedures, these bacteria were not isolated.

It should be emphasized that statements made concerning bacteria isolated from the aquaria refer only to aerobic bacteria and, in particular, to aerobic organisms which could be isolated on nutrient agar. No attempt was made to study anaerobic bacteria in the aquaria.

Approximately the same number of bacterial colonies were isolated from the tank to which no iron was added as from tanks to which 25 mg and 50 mg of ferrous sulfate were added. But in the tank containing 100 mg of iron, ten times as many colonies as in the other three tanks were isolated. This would indicate that the presence of iron might have had some effect on bacteria. But the addition of iron only changed the bacterial concentration in one tank. There was no increase in bacterial population as increasing amounts of iron were added. The addition of iron did not affect bacterial concentration in the same manner it affected the fish growth. The fish growth increased with increasing concentrations of iron, while the bacterial population only increased in the highest iron concentration. It should be emphasized that this conclusion refers only to the total number of bacteria present. The iron could have affected the ratio of different organisms, while the total number remained unchanged. However, from observations of the colony count plates, no marked shift in the ratio of colony types was seen.

The biochemical reactions also indicated that addition of iron had affected the predominant forms of bacteria in the aquaria. But the intensity of this effect was not similar to the effect on the fish. Some bacteria in the iron tanks were biochemically different from the bacteria in the tanks without iron. However, there was not an increasing biochemical difference in the bacteria as iron concentration was raised. Three strains of bacteria in every iron tank differed from their colonial counterparts in the control tank by having the ability to oxidize carbohydrates. But there was no increase in the strains of bacteria which could produce acid from carbohydrates as the iron concentration increased; the same three bacterial types oxidized certain carbohydrates to an acid end-point in all iron tanks.

The purpose of this problem was to determine if a variation occurred in microflora of fish tanks after varying concentrations of iron had been added to the water. If some microbial variation had been detected which changed consistantly with an increase in iron concentration, it might have been possible to hypothesize that a definite relationship existed between iron concentration of water, bacterial population of water, and fish growth. This, however, was not the case. Iron did affect the bacterial population, and the possibility that this bacterial change contributed to the increase in fish growth can not be ruled out. However, since the bacterial variation did not increase with an increase in iron concentration, the bacterial change can not be cited as the predominant factor causing increase in fish growth. Perhaps the iron affected the physiology of the fish directly.

Certain hypotheses may be made as to how iron caused the appearance of three bacterial colonies which could oxidize carbohydrates to an acid end-point. Bacteria identified from the fish aquaria fall into groups which are ill-defined and closely related. Three organisms isolated from the iron tanks were colonially and morphologically identical to three strains isolated from the control aquarium, but they varied in biochemical reactions on certain sugars. Therefore, the three strains from iron water were identified as entirely different species from the three strains in normal water. However, the three strains from the iron tanks might prove to be biochemical variants of three strains from normal water. Variation could have resulted from the induction of a special enzyme system when the colonies were placed in an abnormal environment. When the environment of these water bacteria was changed by the addition of iron to the tanks, an enzyme system, present in the bacteria but non-functional under normal conditions, could have been called into utilization. This new, functional enzyme system could have allowed the bacteria to proliferate in their new environment.

There are two possibilities as to why the induction of an enzyme system catalyzing the oxidation of carbohydrates would be advantageous in the iron tanks. If the action on sugar was aerobic oxidation, the bacteria might also have the ability to oxidize ferrous salts aerobically. The oxidation of ferrous salts would have removed foreign ferrous sulfate from the tank water, for the ferric salt is insoluble.

On the other hand, aerobic oxidation of iron, spontaneously or by some other organisms, might have caused a decrease in the oxygen

concentration of the tanks. In that case, it would have been advantageous for the bacteria to utilize an enzyme system which could supply energy to the cells by a process which would not require oxygen. An environment containing less oxygen could cause the induction of an enzyme system which would oxidize carbohydrates anaerobically. The determination of dissolved oxygen in the fish aquaria did reveal a decrease in the amount of oxygen on the addition of iron to the tanks. In addition, a test run on colonies 3, 9, and 16 from the iron tanks proved that their sugar oxidation was a fermentative process and not aerobic oxidation.

Colonies 3, 9, and 16 from the iron tanks might therefore be variants of colonies 3, 9, and 16 from the control tank. When the oxygen content of the iron tanks was lowered because of the aerobic oxidation of iron, an enzyme system could have been induced in colonies 3, 9, and 16 which allowed them to obtain energy anaerobically.

This type of enzyme induction is also a common occurrence in other forms of life. Yeast cells obtain energy by oxidizing sugars aerobically when they have a plentiful supply of oxygen. These same yeast cells will be induced to obtain energy by anaerobic oxidation or fermentation when they are placed in an environment of low oxygen tension. The same type of reaction takes place in human muscles. Normally, the energy yielding sugar oxidation in muscles is aerobic; as sugar is progressively oxidized, and energy is released and stored as adenosine triphosphate, the released hydrogens are transported to oxygen. In extreme exercise the rate at which energy is needed exceeds the rate at which adenosine triphosphate can be supplied

by the above aerobic process. The inadequacy of aerobic respiration is caused by the slow rate at which oxygen can be transported by blood to the cells in order to remove the free hydrogens. Therefore, the muscle cells transport excess hydrogens to pyruvic acid, one of the products normally formed in the breakdown of glucose, and lactic acid is formed. The muscle cells have changed their energy making process from aerobic to anaerobic oxidation. During rest, the oxygen supply is restored, and the muscles again oxidize sugars aerobically.

Therefore, bacterial changes in fish tanks may be similar to occurrences in yeasts and muscle cells when oxygen tension is lowered in the environment. If this analogy is carried one step further, it can be hypothesized that colonies 3, 9, and 16 would cease to ferment sugars if they were removed from the iron tanks and put back in their normal environment.

As the changes in these bacteria are compared to changes in yeasts and muscle cells, it becomes apparent that this experiment has only illustrated one more example of the similarity of metabolic changes in most forms of life.

BIBLIOGRAPHY

Alexopoulos, Constantine, 1950. Introductory Mycology, John Wiley & Sons, Inc., New York.

Breed, Robert S., E. G. D. Murray, A. Parker Hitchens, 1948. Bergey's Manual of Determinative Bacteriology, sixth edition, The Williams and Wilkins Company, Baltimore.

_____, E. G. D. Murray, Nathan R. Smith, 1957. Bergey's Manual of Determinative Bacteriology, seventh edition, The Williams and Wilkins Company, Baltimore.

Cantarow, Abraham and Bernard Schepartz, 1962. Biochemistry, third edition, W. B. Saunders Company, Philadelphia.

Carpenter, Philip L., 1961. Microbiology, W. B. Saunders Company, Philadelphia.

Difco Manual, 1953. Ninth edition, Difco Laboratories, Incorporated, Detroit.

Henrici, Arthur T., 1933. "Studies of Freshwater Bacteria, I. A Direct Microscopic Technique," Journal of Bacteriology, 25: 277-285.

Herskowitz, Irvin H., 1962. Genetics, Little, Brown and Company, Boston.

Hewitt, L. F., 1950. Oxidation-Reduction Potentials in Bacteriology and Biochemistry, The Williams and Wilkins Company, Baltimore.

Leather, William, Lois D. McIntyre, and S. A. Bralar, 1951. "A Medium for the Study of the Bacterial Oxidation of Ferrous Iron," Science, 114: 280-281.

Levine, Max and H. S. Schoenlein, 1930. A Compilation of Culture Media for the Cultivation of Microorganisms, The Williams and Wilkins Company, Baltimore.

Microbiological Analysis of Water and Milk, 1961. Millipore Filter Corporation, Bedford, Massachusetts.

Pelczar, Michael J. and Roger D. Reid, 1958. Microbiology, McGraw-Hill Book Company, Inc., New York.

Prescott, S. C., C. E. Winslow, and MacH. McCrady, 1946. Water Bacteriology, John Wiley and Sons, Inc., New York.

Roeder, Martin and Rachel Roeder, 1963. "The Effects of Iron on the Growth of Fishes," (abstract), Journal of Elisha Mitchell Scientific Society, 79: 91.

Skerman, V. B. D., 1959. A Guide to the Identification of the Genera of Bacteria, The Williams and Wilkins Company, Baltimore.

Smith, David T., D. X. Martin, N. F. Conant, J. W. Beard, G. Taylor, H. I. Kohn, M. A. Posten, 1948. Zinsser's Textbook of Bacteriology, Appleton-Century-Crofts, Inc., New York.

Standard Methods for the Examination of Water, Sewage, and Industrial Wastes, 1936. Eighth edition, American Public Health Association, New York.

Techniques for Microbiological Analysis, 1961. Millipore Filter Corporation, Bedford, Massachusetts.

Theroux, Frank R., Edward F. Eldridge, and W. LeRoy Mallmann, 1943. Laboratory Manual for Chemical and Bacterial Analysis of Water and Sewage, McGraw-Hill Book Company, Inc., New York.