淡水養魚池の水および底泥における細菌相

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Microflora in the Water and Sediment of Freshwater Culture Ponds*1

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Qualitative and quantitative investigation was performed on the bacterial populations in the water and sediment of the culture ponds rearing carp and goldfish from May 1982 to March 1983. Microflora in the water and sediment was diverse, and major components in the water were *Acientobacter*, *Moraxella*, *Pseudomonas*, *Flavobacterium-Cytophaga*, *Vibrio-Aeromonas*, Enterobacteriaceae, *Bacteroides* type A and other Bacteroidaceae. In addition to these bacteria, major bacteria in the sediment consisted of coryneforms, *Bacillus*, *Micrococcus* and *Clostridium*. Obligate anaerobes were further classified as follows: *Bacteroides*, 15 species; *Fusobacterium*, 3 species; *Clostridium*, 18 species; *Peptococcus*, 1 species; *Peptostreptococcus*, 2 species and unidentified strains. It was suggested that the difference of the microflora between glass aquaria and culture ponds may mainly depend on the diversity of the environment for bacterial populations.

Total aerobic bacteria including facultative anaerobes were 10^3 to 10^5 ml^{-1} and 10^6 to 10^7 g^{-1} and total anaerobic bacteria were $<2\times10^1$ to 10^4 ml^{-1} and 10^3 to 10^6 g^{-1} in the water and the sediment, respectively. Total anaerobic bacteria were found to correlate with water temperature or COD.

Intensive fish farming utilizes a minimum of land and water resources while providing an economically important source of high quality of protein. Accordingly, large amounts of organic matter originated from the excess feed, excreta and dead bodies of aquatic organisms is always supplied to pond water and accumulates finally on the pond bottom. The organic matter is decomposed mainly by heterotrophic bacteria into inorganic matter including carbon dioxide, ammonia and phosphate, and organic matter including vitamins. These substances may stimulate the growth of phytoplankton and result in the water bloom known as "Aoko". When anoxic layer is formed in the bottom water after rapid consumption of dissolved oxygen by bacteria, deleterious substances such as sulphides and methane gas which are known to decrease both the growth of the fish and the ultimate yield of ponds, are porduced.[1)]

For the purpose of evaluating fish farming condition, physico-chemical works have been reported but microbiological approach has been rarely performed.[2-5)] In the course of this study the authors intend to study the ecology of heterotrophic bacteria in freshwater culture ponds to grasp the culture environments.

In this paper qualitative and quantitative investigation was performed on bacterial populations in the water and sediment of stagnant water ponds rearing carp *Cyprinus carpio* and goldfish *Carassius auratus*.

Materials and Methods

Culture Ponds

The stagnant water ponds rearing carp and goldfish are ca. 2,500 m^2 and 150 m^2, respectively and both are located in Tokyo Metropolitan Fisheries Experimental Station, Mizumoto, Tokyo. About 5,600 kg of carp (average body weight, 7,000 g) and 37 kg of glodfish (average body weight, 160 g) were maintained and fed artificial diets, mainly a pellet diet. Water in both ponds were simultaneously supplied with top and well waters, and bottom sediments are composed of sandy mud in the carp-culture pond and mud in the goldfish-culture pond. Water depth of both ponds were 70 to 100 cm.

The investigation was carried out on May 20, July 16, September 27, November 11, 1982, January 19 and March 4,1983. Middle water was collected with a KITAHARA's water sampler (Rigosha) and sediment was sampled with a EKMAN-BERGE bottom sampler (Rigosha). Collected samples

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were transferred to sterile test tubes with rubber stoppers, cooled on ice and transported to the laboratory within 4 h.

Chemical Analysis

Water temperature (WT) was measured *in situ* with a mercury thermometer. Dissolved oxygen (DO) was measured by WINKLER's method, and pH was electronically determined by a pH meter, HM-5A (Toa electronics). Chemical oxygen demand (COD) was measured by MATSUE.[6] Ammonia (NH_4-N) was determined by NIMURA's method,[7] and nitrite (NO_2-N) and nitrate (NO_3-N) were measured according to STRICKLAND and PARSONS.[8]

Enumeration of Bacteria

The sediment was weighed, transferred into a sterile test tube with a suitable amount of diluent to effect a tenfold dilution. The diluent used is that reported by MITSUOKA[9] as the dilution solution A.

The water and sediment samples were then vigorously vibrated with a Vortex mixer for 1 to 2 min, diluted serially and plated onto 9 different agar media. The inoculated media included 1/20 PYBGF agar,[10] PYBGF agar,[10] PEA blood agar (BBL), MacConkey agar (Eiken), EG blood agar (Nissui), NBGT-1/3S blood agar,[11] modified FM agar (Nissui), Bacteroides agar (Nissui) and FM-CW blood agar (Eiken).

The inoculated 1/20 PYBGF and PYBGF agars were incubated at 20°C for 5 to 7 days, and PEA and MacConkey agars at 37°C for 2 to 3 days, both under aerobic condition. The inoculated EG, NBGT-1/3S, FM, Bacteroides and FM-CW agars were incubated at 30°C for 5 to 7 days under anaerobic condition as the authors[12,13] found that almost all the obligate anaerobes isolated from the river and rivermouth are able to grow at 20 to 30°C. Anaerobiosis was established by evacuating the atmosphere of an anaerobic jar containing steel wool which was activated by an acidic sulfate solution and replacing the atmosphere with carbon dioxide gas.[9] After incubation, colonies that appeared on each plate were counted and viable count (*i.e.*, colony forming unit) per m*l* or g was obtained.

Identification of Bacteria

After the viable count was determined, colonies were divided into some types according to colonial characteristics. Three representatives of each colony type were streaked onto 1/20 PYBGF, PYBGF or EG agars, and purified by a second plating onto the same medium.

Identification of the aerobes and facultative anaerobes isolated under aerobic condition was facilitated by examination of pigmentation of the colony and the shape, spore formation, arrangement, staining characteristics and motility of the cells. In addition, the ability of the isolates to produce oxidase (KOVACS method[14]) and catalase, to grow under anaerobic condition, and to metabolize glucose fermentatively or oxidatively using OF medium (BBL) was tested. Gram-negative bacteria were identified by generic level by the modified scheme[15] of SHEWAN *et al.*[16] and gram-positive bacteria were identified according to COWAN.[17]

The anaerobes including facultative anaerobes isolated under anaerobic condition were classified on the basis of gram reaction, cellular morphology, spore formation, cellular arrangement and the ability to grow under aerobic condition.

The representative strains of obligate anaerobes were further identified by specific level by Minitek Numerical Identification System (BBL).

Results

Water Quality of Culture Ponds

Water quality and microflora in the water and sediment of stagnant water ponds rearing carp and goldfish were bimonthly investigated. The results of chemical analysis in the middle water of culture ponds in the period of May 1982 to March 1983 are summarized as follows: Water temperature was ranged from 5.2 to 23.8°C and from 4.6 to 24.4°C; DO, 7.7 to 12.5 ppm and 6.6 to 10.3 ppm; pH, 7.6 to 9.4 and 7.5 to 10.0; COD, 5.3 to 9.7 ppm and 6.6 to 10.7 ppm; NH_4-N, 0.01 to 0.35 ppm and 0.00 to 1.61 ppm; NO_2-N, 0.01 to 0.03 ppm and 0.00 to 0.07 ppm; and NO_3-N, 0.01 to 0.60 ppm and 0.02 to 1.05 ppm in the carp-culture pond and the goldfish-culture pond, respectively.

Water bloom was observed in May and July in both the culture ponds. Although the surface and middle water contained high concentrations of dissolved oxygen at any time in both the ponds, the anoxic or low dissolved oxygen layers (less than 1 ppm) occured in the bottom water in the summer.

Viable Counts of Bacteria

The number of viable bacteria recovered when

Table 1. Colony counts (log No. ml^{-1}) of the bacteria belonging to different components in the water of carp-culture pond

Component	May 20	Jul 16	Sep 27	Nov 11	Jan 19	Mar 4
Aerobes						
Acinetobacter	2.3	—	3.2	3.2	2.3	2.3
Moraxella	—*	—	2.3	—	1.3	1.3
Pseudomonas	1.8	1.3	4.0	2.3	3.8	4.0
Enterobacteriaceae	2.1	1.3	3.3	2.6	—	1.8
Vibrio-Aeromonas	3.3	2.8	3.7	3.6	2.8	3.3
Flavobacterium	2.7	—	3.5	3.3	2.3	2.3
Coryneforms	1.6	1.6	2.3	1.3	1.3	1.3
Bacillus	1.8	2.7	2.3	1.9	2.3	1.6
Staphylococcus	1.6	2.8	2.8	1.3	—	—
Micrococcus	—	—	2.8	1.3	—	—
Chromobacterium	—	—	—	—	—	1.3
Anaerobes						
Clostridium	1.3	1.3	1.3	1.6	—	1.6
Bacteroides type A	3.0	2.6	3.3	3.0	—	—
Other Bacteroidaceae	1.3	1.3	3.2	—	—	1.6
Spirochetes	—	1.3	—	—	—	—
Total viable count	3.6	3.4	4.5	4.0	3.9	4.1

* Not detected.

the water and sediment of culture ponds were sampled, was measured. Different numbers of viable counts were observed on 9 different agar media in each sample as shown as follows: The number of viable counts on PYBGF agar was ranged from 10^2 to 10^4 ml^{-1} and 10^5 to 10^7 g^{-1}; 1/20 PYBGF agar, 10^3 to 10^5 ml^{-1} and 10^5 to 10^7 g^{-1}; PEA agar, $<2\times10^1$ to 10^2 ml^{-1} and 10^4 to 10^5 g^{-1}; MacConkey agar, 10^1 to 10^4 ml^{-1} and 10^4 to 10^6 g^{-1}; EG agar, 10^2 to 10^4 ml^{-1} and 10^3 to 10^6 g^{-1}; NBGT-1/3S agar, $<2\times10^1$ to 10^3 ml^{-1} and 10^4 to 10^6 g^{-1}; modified FM agar, $<2\times10^1$ to 10^3 ml^{-1} and $<2\times10^2$ to 10^5 g^{-1}; Bacteroides agar, $<2\times10^1$ to 10^3 ml^{-1} and 10^2 to 10^5 g^{-1}; and FM-CW agar, $<2\times10^1$ to 10^3 ml^{-1} and 10^3 to 10^4 g^{-1} in the water and sediment, respectively. The largest number of viable counts was observed on 1/20 PYBGF and PYBGF agars incubated under aerobic condition and EG agar under anaerobic condition. In the water samples, the agar medium with lower nutrients (1/20 PYBGF) showed, in some degree, larger number of viable counts. The number of viable counts in the water of the goldfish-culture pond was larger than that of the carp-culture pond.

Generic Composition of Bacteria

One thousand three hundred and eighty-seven strains of aerobic and facultatively anaerobic bacteria were isolated from the water and sediment of culture ponds under aerobic condition and identified by generic level. All strains were classified into 13 groups and major components were *Vibrio-Aeromonas* group (23.7%), *Bacillus* (13.5%), *Pseudomonas* (13.0%), coryneforms (10.2%), *Flavobacterium-Cytophaga* group (8.4%) and Enterobacteriaceae (8.0%).

Similarly, one thousand and forty-three strains isolated under anaerobic condition were classified into 10 groups and 36.8% isolates were facultative anaerobes. Obligate anaerobes were mainly composed of *Bacteroides* type A (21.9%), other Bacteroidaceae (18.2%) and *Clostridium* (16.5%).

Microflora in the Water of Culture Ponds

The maximum viable counts of bacteria belonging to different genera in the water of carp-culture pond and goldfish-culture pond from May 1982 to March 1983 were shown in Tables 1 and 2.

In the carp-culture pond, total viable counts (TVC) which were calculated by summing up the maximum viable counts of bacteria belonging to different genera were 10^3 to 10^4 ml^{-1}, total aerobic bacteria including facultative anaerobes were 10^3 to 10^4 ml^{-1} and total anaerobic bacteria were $<2\times10^1$ to 10^3 ml^{-1}. Major components were *Acinetobacter*, *Pseudomonas*, Enterobacteriaceae, *Vibrio-Aeromonas*, *Flavobacterium-Cytophaga* and *Bacteroides* type A (Table 1). *Bacteroides* type A, however, was not detected in the winter. Although no good correlation of TVC or total aerobic bacteria with water temperature (WT) or COD was

Table 2. Colony counts (log No. ml^{-1}) of the bacteria belonging to different components in the water of goldfish-culture pond

Component	May 20	Jul 16	Sep 27	Nov 11	Jan 19	Mar 4
Aerobes						
Acinetobacter	3.0	1.9	5.4	2.6	3.6	2.3
Moraxella	2.3	1.9	4.6	3.3	3.3	4.5
Pseudomonas	2.9	2.3	3.3	2.3	4.5	4.8
Enterobacteriaceae	4.0	4.2	4.5	2.6	2.3	1.3
Vibrio-Aeromonas	4.3	4.0	4.4	4.4	—	3.0
Flavobacterium	2.8	3.0	4.1	2.8	2.8	3.2
Coryneforms	—*	3.0	3.0	2.3	2.3	1.3
Bacillus	—	3.4	1.8	2.3	1.8	2.3
Streptococcus	—	1.9	3.0	1.3	—	—
Staphylococcus	1.8	2.6	1.3	2.3	—	—
Micrococcus	2.1	1.8	1.3	1.9	1.3	—
Anaerobes						
Clostridium	2.3	2.3	2.6	1.8	—	1.6
Bacteroides type A	—	2.0	2.6	1.8	—	1.8
Other Bacteroidaceae	2.4	3.1	3.8	—	—	1.4
Gram-positive cocci	—	4.0	2.3	1.9	—	—
Total viable count	4.5	4.6	5.6	4.4	4.6	5.0

* Not detected.

observed, total anaerobic bacteria (TAN) showed positive correlation with WT or COD as follows:

$\log TAN_{carp\text{-}Pond}=0.99+0.10 \cdot WT$, $r=0.86$

$\log TAN_{carp\text{-}Pond}=-0.44+0.39 \cdot COD$, $r=0.84$

where the value of log TAN in January was calculated as 1.2.

In the goldfish-culture pond, TVC, total aerobic bacteria and total anaerobic bacteria were 10^4 to 10^5 ml^{-1}, 10^4 to 10^5 ml^{-1} and $<2\times10^1$ to 10^4 ml^{-1}, respectively. *Moraxella, Clostridium,* Bacteroidaceae and anaerobic cocci predominated in addition to the bacteria detected in the carp-culture (Table 2). Obligate anaerobes were rarely detected in the winter. Total anaerobic bacteria were also correlated positively with WT or COD as follows:

$\log TAN_{goldfish\text{-}Pond}=0.54+0.14 \cdot WT$, $r=0.98$

$\log TAN_{goldfish\text{-}Pond}=-0.74+0.38 \cdot COD$, $r=0.58$

where the value of log TAN in January was calculated as 1.2. TVC and total aerobic bacteria were not significantly correlated with WT or COD as similar to the carp-culture pond.

Microflora in the Sediment of Culture Ponds

As presented in Table 3, TVC, total aerobic bacteria and total anaerobic bacteria in the sediment of carp-culture pond from May 1982 to March 1983 were 10^6 g^{-1}, 10^6 g^{-1} and 10^3 to 10^5 g^{-1}, respectively. Major components were coryneforms, *Bacillus*, *Clostridium* and Bacteroidaceae in addition to the bacteria present in the water. Comparing with TVC or total aerobic bacteria, total anaerobic bacteria showed relatively high correlation with WT as follows:

$\log TAN_{carp\text{-}Pond}=4.11+0.06 \cdot WT$, $r=0.63$.

As presented in Table 4, TVC, total aerobic bacteria and total anaerobic bacteria in the sediment of goldfish-culture pond were 10^6 to 10^7 g^{-1}, 10^6 to 10^7 g^{-1} and 10^4 to 10^6 g^{-1}, respectively. *Streptococcus, Staphylococcus* and anaerobic cocci were predominant in addition to the bacteria detected in the carp-culture pond.

The relatively high correlation of WT was not observed with TVC or total aerobic bacteria but with total anaerobic bacteria as follows:

$\log TAN_{goldfish\text{-}Pond}=4.25+0.07 \cdot WT$, $r=0.76$

Specific Composition of Obligate Anaerobes

Of obligate anaerobes isolated from the water and sediment of culture ponds rearing carp and goldfish, 144 strains of Bacteroidaceae, 83 strains of clostridia and 22 strains of anaerobic cocci were selected and identified. As presented in Table 5, Bacteroidaceae was composed of 15 species of genus *Bacteroides* and 3 species of genus *Fusobacterium.* As expected, the strains identified as *Bacteroides* type A could not be identified according to Minitek System. Clostridia consisted of 18 species of genus *Clostridium* and 13 unidentified strains. Anaerobic cocci were composed of *Peptococcus saccharolyticus*, *Peptostreptococcus anaero-*

Table 3. Colony counts (log No. g^{-1}) of the bacteria belonging to different components in the sediment of carp-culture pond

Component	May 20	Jul 16	Sep 27	Nov 11	Jan 19	Mar 4
Aerobes						
Acinetobacter	4.3	—	6.1	5.6	5.3	—
Moraxella	5.3	—	—	—	4.3	—
Pseudomonas	5.5	—	6.1	4.9	5.4	4.3
Enterobacteriaceae	—*	3.6	6.6	5.1	—	4.5
Vibrio-Aeromonas	5.9	4.7	6.6	6.2	5.6	6.2
Flavobacterium	4.1	—	5.1	4.3	5.0	4.9
Coryneforms	4.3	3.6	5.3	5.5	5.3	4.8
Bacillus	4.3	5.8	4.8	4.3	5.6	3.6
Streptococcus	—	—	4.3	3.3	3.3	—
Staphylococcus	4.0	5.7	5.8	3.3	3.3	—
Micrococcus	3.8	4.8	—	4.5	—	4.3
Anaerobes						
Clostridium	3.0	4.3	5.1	3.8	3.3	5.1
Bacteroides type A	4.8	5.2	5.1	5.3	3.1	3.9
Other Bacteroidaceae	2.3	5.1	5.2	3.9	3.3	5.3
Gram-positive cocci	—	—	2.6	—	—	—
Total viable count	6.2	6.3	6.9	6.5	6.2	6.4

* Not detected.

Table 4. Colony counts (log No. g^{-1}) of the bacteria belonging to different components in the sediment of goldfish-culture pond

Component	May 20	Jul 16	Sep 27	Nov 11	Jan 19	Mar 4
Aerobes						
Acinetobacter	5.4	—	6.5	4.9	—	3.6
Moraxella	3.3	—	4.8	6.6	3.3	3.3
Pseudomonas	6.0	3.6	5.6	6.1	6.1	6.5
Enterobacteriaceae	4.3	—	4.4	5.9	4.3	—
Vibrio-Aeromonas	5.6	4.5	5.0	4.8	6.1	6.1
Flavobacterium	4.6	5.3	5.6	4.3	5.1	5.6
Coryneforms	4.9	5.1	6.9	5.1	4.9	4.7
Bacillus	5.1	5.8	5.8	5.4	—	4.6
Streptococcus	5.6	6.1	6.7	4.3	—	—
Staphylococcus	—*	4.2	6.8	—	4.2	4.3
Micrococcus	3.8	4.3	—	—	4.6	—
Anaerobes						
Clostridium	4.4	6.1	4.9	4.8	4.3	4.9
Bacteroides type A	3.1	2.6	3.3	4.0	—	4.1
Other Bacteroidaceae	5.0	5.8	5.1	5.0	3.9	4.9
Gram-positive cocci	2.8	6.0	4.8	—	—	—
Total viable count	6.3	6.8	7.4	6.8	6.8	6.7

* Not detected.

bius, Ps. productus, Stretococcus intermedius and 10 strains of unidentified gram-positive cocci.

Major species were *Bacteroides* type A, *B. assacharolyticus, B. ovatus, B. ruminicola, B. uniformis, Cl. bifermentans* and *Cl. butyricum.* The detection of each species was scattered, and a defined tendency was not recognized in the seasonal changes of each viable count.

Discussion

In the previous paper,[3] we examined the microflora in the water of glass aquaria rearing carp and found that *Vibrio-Aeromonas* group and *Bacteroides* type A, which are the dominant components in the intestinal tracts of freshwater fishes, became predominant. It was suggested from the above

Table 5. Specific composition of obligate anaerobes isolated from the water and sediment of culture ponds

Species	Water	Sediment
Bacteroides asaccharolyticus	11	3
B. biacutus	1	3
B. bivius	0	1
B. capillosus	1	8
B. distasonis	1	4
B. eggerthii	0	1
B. fragilis	2	1
B. melaninogenicus ss. *melaninogenicus*	1	4
B. oralis	2	1
B. ovatus	2	10
B. ruminicola	3	9
B. thetaiotaomicron	0	1
B. uniformis	8	4
B. group 3425A	3	1
B. type A	10	42
Clostridium beijerinckii	0	1
Cl. bifermentans	7	8
Cl. botulinum	0	3
Cl. butyricum	0	20
Cl. cadaveris	0	1
Cl. chauvoei	2	0
Cl. clostridiiforme	0	4
Cl. difficile	1	0
Cl. novyi	1	2
Cl. perenne	0	1
Cl. perfringens	0	1
Cl. sordelli	0	3
Cl. septicum	0	1
Cl. sphenoides	0	2
Cl. sporogenes	0	2
Cl. symbiosum	0	2
Cl. tertium	0	1
Cl. tetani	0	7
Cl. spp.	4	9
Fusobacterium mortiferum	0	1
F. necrogenes	1	2
F. prausnitzii	1	1
Peptococcus saccharolyticus	0	2
Peptostreptococcus anaerobius	1	0
Ps. productus	1	1
Streptococcus intermedius	3	4
unidentified gram-positive cocci	7	3
Total	74	175

result that the excretion of bacteria from the fish, especially as fecal forms, effects significantly on the microflora of water.

The results obtained in the present study indicate that total obligate anaerobes in the culture ponds fluctuated with water temperature or organic matter (as COD) in spite of no correlation between total aerobes and water temperature or COD. This seems that temperature and organic matter are limitting factors for the growth of obligate anaerobes in the culture ponds investigated. It, however, seems likely that the high temperature stimulates the activity of fish and results in the increases of feed intake and of feces excretion. These speculation will be supported by the fact that *Vibrio-Aeromonas* group and *Bacteroides* type A fluctuated with water temperature, especially in the goldfish-culture pond. In the regression equations between total anaerobic bacteria and water temperature, the values of slope in the water showed higher than those in the sediment. This result suggests that the increase of water temperature promotes the fish to swim actively and blow up the bottom sediment containing bacteria and that the rate of bacterial increase to water temperature in the pond water becomes finally higher than that in the sediment.

Many obligate anaerobes other than *Bacteroides* type A were also isolated from the water and sediment of culture ponds. We think that obligate anaerobes originate from the terrestrial soil or feces of homeotherm invaded and accumulate on the bottom sediment. Once the anoxic layer is formed after rapid consumption of dissolved oxygen to decompose large amounts of organic matter, these organisms may proliferate. This would be supported by the fact that many anaerobes isolated distributes in the soil, waste and animal intestines as reported by HOLDEMAN *et al.*[18]

The present findings shows that the microlfora in the water and sediment of culture ponds is diverse, compared with that in glass aquaria.[3] The difference in the microflora of both the systems may depend on that in the diversity of environments for the bacterial populations. The glass aquarium consistes of glass, water and sand as filter materials and macroorganisms are restricted in the system and particulate materials are removed with filter, whereas the culture pond is composed of many complicated factors unable to analyze easily. Especially, the water bloom may significantly affect on the bacterial populations by excreting promoting and inhibiting substances and offering microenvironments. Thus, the combination of field observation and modeling systems should be required to analyze the ecology of heterotrophic bacteria in the culture ponds.

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