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Evaluation of the microbial population from recirculating marine aquarium

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Abstract. Marine ornamental fishes are the most beautiful and fascinating creatures of the aquatic environment. Ornamental fish keeping has developed into a hobby and ranks next to photography. These fishes are facing more problems from microbial pathogens. To prevent disease outbreaks, it is important to understand how diseases are occurring. Therefore an attempt was made to study the bacterial population, pathogenic bacteria identification and nitrogen concentration in the aquarium waters. The observed ammonia values ranged from 0.031 to 0.144 Mg L^{-1}, nitrite from 0.014 to 0.314 Mg L^{-1} and nitrate from 0.793 to 69.821 Mg L^{-1}. The total bacterial counts of the water samples ranged from 5.4 x 10^2 to 2.1 x 10^7 CFU mL^{-1}. The maximum bacterial count was found in the storage tank water sample (2.1 x 10^7 CFU mL^{-1}) and the minimum bacterial count was found in the healthy tank sample (5.4 x 10^2 CFU mL^{-1}). The most dominant colonies were *Escherchia coli, Vibrio* sp., *Pseudomonas* sp., *Enterobacter* sp. and *Bacillus* sp. Among these, diseased aquarium tank microbes used for the further identification. Of these the most predominant species (*Vibrio parahaemolyticus, Bacillus subtilis* and *Bacillus licheniformis*) were identified based on the MIS gas chromatography.
Key words: marine aquarium, disease, microbes, ornamental fishes, MIS gas chromatography.

Introduction. Aquarium fishes are susceptible to infectious and non-infectious diseases. Infectious diseases are caused by various biological organisms such as bacteria, virus, fungi or protozoan which ultimately, reproduce and spread to other fishes in the tanks. Non-infectious diseases are non-transmissible and can be caused by a variety of factors such as poor water quality and poor nutrition. Bacterial diseases were observed very frequently in the ornamental fish species kept in aquarium tanks in addition to fin rot, white spot and local infections in traumatic injuries.

Stocking densities in aquaria are limited primarily by the dissolved oxygen content and often, also by the ammonia (NH_3) concentrations (Meade 1985). Ammonia is formed as the end product from protein metabolism of fish. Fish expel NH_3 through their gills by branchial diffusion. Ammonia and ammonium originating from the gills comprise 60–90% of the total nitrogen excreted by fish (Forster & Goldstein 1969; Rychly 1980). Urea is also expelled through the gills and accounts for 9–27% of the soluble nitrogen excreted (Clark et al 1985).

Another source of ammonia in aquaria is microbial ammonification of organic nitrogen due to feed wastage and fish excreta. This particulate matter accounts for 3.4–4.2% of the total nitrogenous waste in tank systems (Clark et al 1985). Ammonia exists in water in two forms, i.e., as ionized ammonium ions (NH_4^+) and unionized ammonia. The two forms together are indicated as total ammonium nitrogen (TAN). Unionized ammonia is more toxic form than ionized ammonia. Levels above 0.1 mg NH_3-N L^{-1} are considered detrimental to fish (Van Rijn et al 1990) and in practical terms, fish should not be chronically exposed to NH_3-N levels of more than 50 µg N L^{-1} (Frances et al 2000).

Nitrification is the process in which ammonium is oxidized to nitrate in two steps carried out by two different groups of chemolithoautotrophic bacteria. In the first step, ammonium-oxidizing bacteria oxidize ammonium to nitrite, which is converted to nitrate by nitrite-oxidizing bacteria in the second step (Focht & Verstraete 1977). Therefore,

nitrification is an important process in aquaria, since it can convert toxic ammonia to nitrate, which is relatively harmless to fish and can be kept at safe levels through regular water changes (Hargreaves 1998). Nitrite is formed in aquaria from the oxidation of ammonia by chemolithoautotrophic ammonium-oxidizing bacteria, but it can also be formed as a consequence of denitrification activity in anoxic zones in the biofilter. The accumulation of nitrite can be toxic to fish and other aquatic organisms. Nitrite reacts with hemoglobin to form methemoglobin inhibiting the transport of oxygen resulting in methemoglobinemia or brown blood disease (Frances et al 1998). Because of the ability to remove the ammonium ion and nitrite from water, the ammonium-oxidizing bacteria and nitrite-oxidizing bacteria play an essential role in aquaria and aquaculture systems. Therefore, these systems usually provide a solid matrix, often called a biological filter, to promote the growth of these bacteria (Wheaton et al 1994).

Bacterial disease is the most common problem in ornamental fishes. The majority of bacterial fish pathogens are natural inhabitants of the aquatic environment, whether it is freshwater or marine (Gregory et al 2001). Bacteria may be the primary cause of disease, or they may be secondary invaders, taking advantages of a branch in the fish's integument or compromise of its immune system. Nearly every bacterial pathogen of fish is capable of living independently away from the fish host (Inglis et al 1993).

Generally, organic matter in recirculation aquariums is derived from uneaten feed or diets, dead bodies, wounded fishes and excreta of fish. These inorganic matters can directly affect total bacterial populations of the aquaria. The water quality of recirculating system is mainly affected by the bacterial flora in the water column and filter materials as well as the different elements of this closed system. The low efficiency of biological filter media causes the bloom of opportunistic pathogenic bacteria in the rearing tanks (Blancheton & Canaguier 1995). The exhibition aquaria and closed aquaculture systems reproduce aquatic environments with high densities of fish and the fishes kept in such intensive systems are very sensitive to the microbial flora of the water (Blanch et al 1999). Moreover, bacterial populations in aquaculture facilities and in exhibition aquaria may differ from those of the natural environment and may affect the nutrition and health of the fish. The influence of associated bacteria in water, food and initial stages of fish in captivity has been demonstrated by Nicolas et al (1989). It is apparent that the presence of potentially pathogenic microorganisms and opportunistic pathogenic bacteria in these aquaria would present a risk to the public health. The knowledge about these aquarium pathogenic organisms is important to hobbyist. Thus, the present study was initiated to qualitatively and quantitatively determine the bacterial population in the water supplied with ornamental fishes.

Infected fish alter their normal behavioral patterns in response to a disease agent or environmental changes. Often the sign observed is not specific, but indicative of either infectious disease or non-infectious, water quality or nutrition related problems. To prevent disease outbreaks, it is important to understand how diseases are occurring. Therefore an attempt was made to study the bacterial population, pathogenic bacteria identification and nitrogen concentration in the aquarium waters.

Materials and Methods

The water samples were collected from the aquarium at every month. Ammonia, nitrite (NO_2^-) and nitrate (NO_3^-) were analyzed spectrophotometrically using Hitachi 220S model UV-VIS spectrophotometer following the methods described by Grasshoff et al (1983) with suitable modifications (C-MARS 2002).

Bacterial Population. Collection of Water Samples. The water samples were collected from both healthy and diseased aquarium and from the storage tank in well cleaned, dried and sterile bottle for bacteriological examination. The water samples were collected before and after application of probiotics. All the samples were analyzed within hour of collection. The sample was then transferred to a sterile conical flask (150 mL) containing 99 mL of sterile diluents, and serial dilution was performed to get 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} suspension samples. For enumeration of Total Heterotrophic Bacteria

(THB), Zobell marine agar medium (Hi-media, Mumbai) was used. For enumeration of *Vibrio* spp. TCBS media was obtained from Hi-media, Mumbai.

Enumeration of the microbes was done by adopting spread plate method. In this method, sterile media were poured into Petri dishes aseptically and allowed to solidify. 0.1 mL of the diluted sample was pipette out into sterile Petri-dish. It was made spread in the plate first by rotating it in clockwise and then anti-clockwise directions for three times and then spread with the help of a 'L'-rod. The plates were incubated in an inverted position at 28±2°C. All the determinations were carried out in triplicates. After the incubation period of 2 to 3 days, the colonies were counted. The plates were examined and counted the number of colonies per plate. The microbial load in the given sample was calculated using the following formula and it is expressed as Colony Forming Units (CFU) per gram of the sample. The predominant microbial colonies were identified based on the MIS methods.

Microbial Identification System (MIS). The MIS comprises of a Gas Chromatography with following configuration. Few loopful (approximately 40 mg of cells) of live wet cells were harvested from the plate culture. The harvested cells were transferred to a clean dry Teflon lined screw cap tube and labeled. A strong methanolic base combined with heat and pressure kills and lyses the cells. Fatty acids were cleaved from the cell lipids and converted to their sodium salts. This was performed by adding 1 mL of reagent 1 to each sample tube, mixed well and kept in a water bath for 30 minutes at 100°C. Immediately after saponification the tubes were brought back to room temperature by keeping them in a cold water bath. Methylation converts the fatty acids (as sodium salts) to fatty acid methyl esters which increases the volatility of the fatty acids for GC analysis. This was achieved by adding 2 mL of reagent 2 to each tube, mixed well and kept in a water bath at 80°C for 10 minutes. Fatty acid methyl esters were removed from the acidic aqueous phase and transferred to an organic phase with a liquid-liquid extraction procedure. The 1.25 mL of reagent 3 was added to each tube, mixed well for 10 minutes and the lower aqueous phase was removed using a Pasteur pipette. A mild base solution was added to the sample tubes to remove fatty acids and residual reagents from the organic extract. Residual reagents will damage the chromatographic system resulting in tailing and loss of the hydroxyl fatty acid methyl esters. The 3 mL of reagent 4 was added to each tube, mixed well for 5 minutes and centrifuged at 2000 rpm for 3 minutes. The upper solvent phase was removed and stored in GC vial for analysis.

Results

Nitrogen Load. The water samples were collected from both healthy and diseased aquarium and from the storage tank at every ten days interval and the concentration of ammonia, nitrite and nitrate were estimated spectrophotometrically as per the standard procedures (Grassholf et al 1983; C-MARS 2002) and the results are given in Table 1 and Fig. 1 to 3.

Bacterial Population. The total bacterial counts of the water samples were ranged from 5.4×10^2 to 2.1×10^7 CFU mL^{-1}. The maximum bacterial count was found in the storage tank water sample (2.1×10^7 CFU mL^{-1}) and the minimum bacterial count was found in the healthy tank sample (5.4×10^2 CFU mL^{-1}, Table 2).

Table 1

Nitrogen load in aquarium tank

Days	*Ammonia ($\mu g\ L^{-1}$)*	*Nitrite ($\mu g\ L^{-1}$)*	*Nitrate ($\mu g\ L^{-1}$)*
Initial	0.031	0.014	0.793
10	0.038	0.081	5.145
20	0.036	0.119	12.456
30	0.035	0.125	15.289
40	0.061	0.136	21.728
50	0.064	0.145	23.542
60	0.079	0.156	35.624
70	0.086	0.164	39.148
80	0.088	0.186	39.753
90	0.104	0.205	44.821
100	0.133	0.235	54.167
110	0.144	0.314	69.821

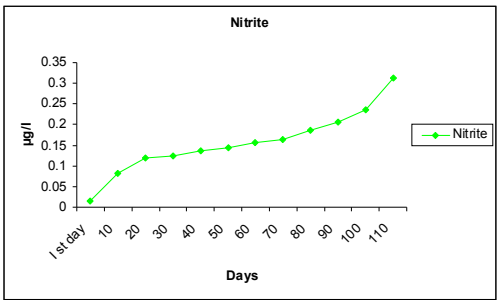

Figure 1. Ammonia concentration

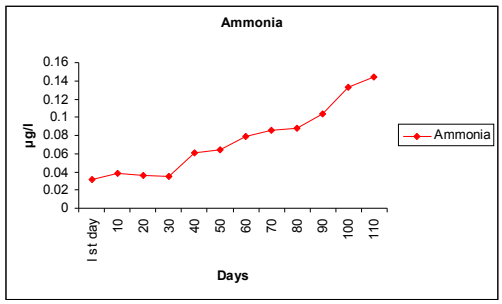

Figure 2. Nitrite concentration

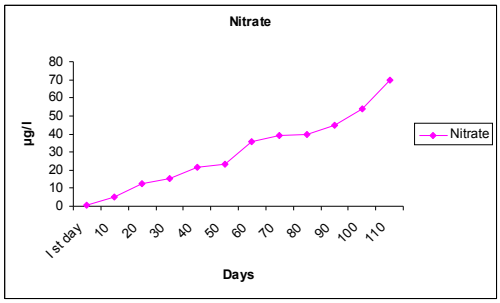

Figure 3. Nitrate concentration

Table 2

Total bacterial population in different samples from aquarium

S.No	*Healthy tank*	*Diseased tank*	*Storage tank*
Ist day	5.4x 10^2	3.2x10^3	28.3x10^3
10	25.6x10^2	24.3x10^3	50.2x10^3
20	38.3x10^2	65x10^3	73.2x10^3
30	72.5x10^2	83.2x10^3	1.23x10^4
40	2.8x10^3	11.6x10^4	41x10^4
50	28x10^3	43.2x10^4	86x10^4
60	53x10^3	2.86x10^5	12.82x10^5
70	70.2x10^3	35x10^5	43.9x10^5
80	8.9x10^4	72x10^5	78.6x10^5
90	28x10^4	3.6x10^6	38.2x10^6
100	41x10^4	21x10^6	76.2x10^6
110	61x10^4	41x10^6	2.1x10^7

Bacterial Identification. The selected colonies were identified based on the biochemical characters. The most dominant colonies were *Escherchia coli, Vibrio* sp., *Pseudomonas* sp., *Entrobacter* sp. and *Bacillus* sp. The only diseased aquarium tank microbes used for the further identification. Among these, three of the most predominant species (*Vibrio parahaemolyticus, Bacillus subtilis* and *Bacillus licheniformis*) were identified based on the MIS gas chromatography. The chromatograms of the species identified are shown in Fig. 4 to 6.

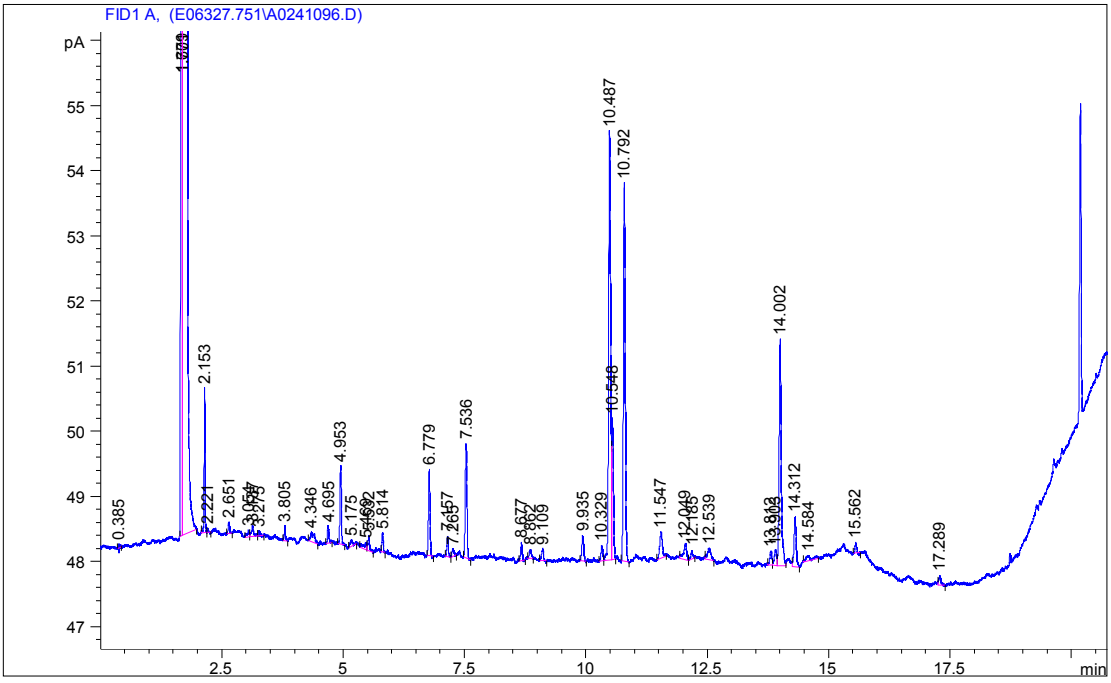

Figure 4. Chromatogram of *Vibrio parahaemolyticus*

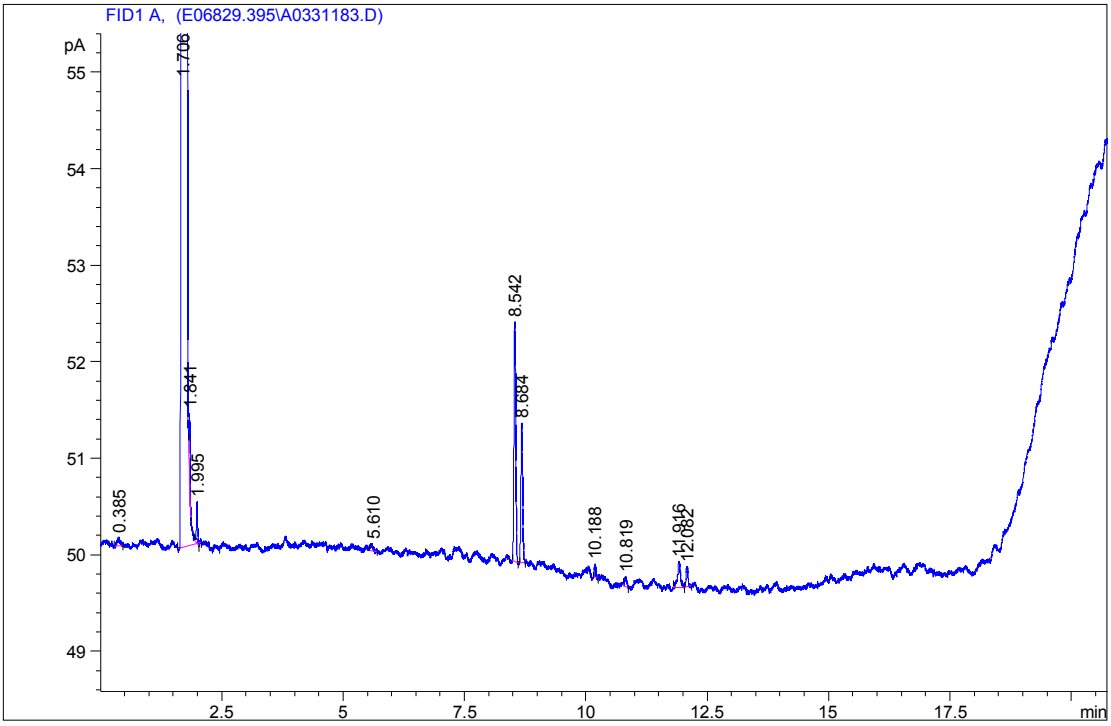

Figure 5. Chromatogram of *Bacillus subtilis*

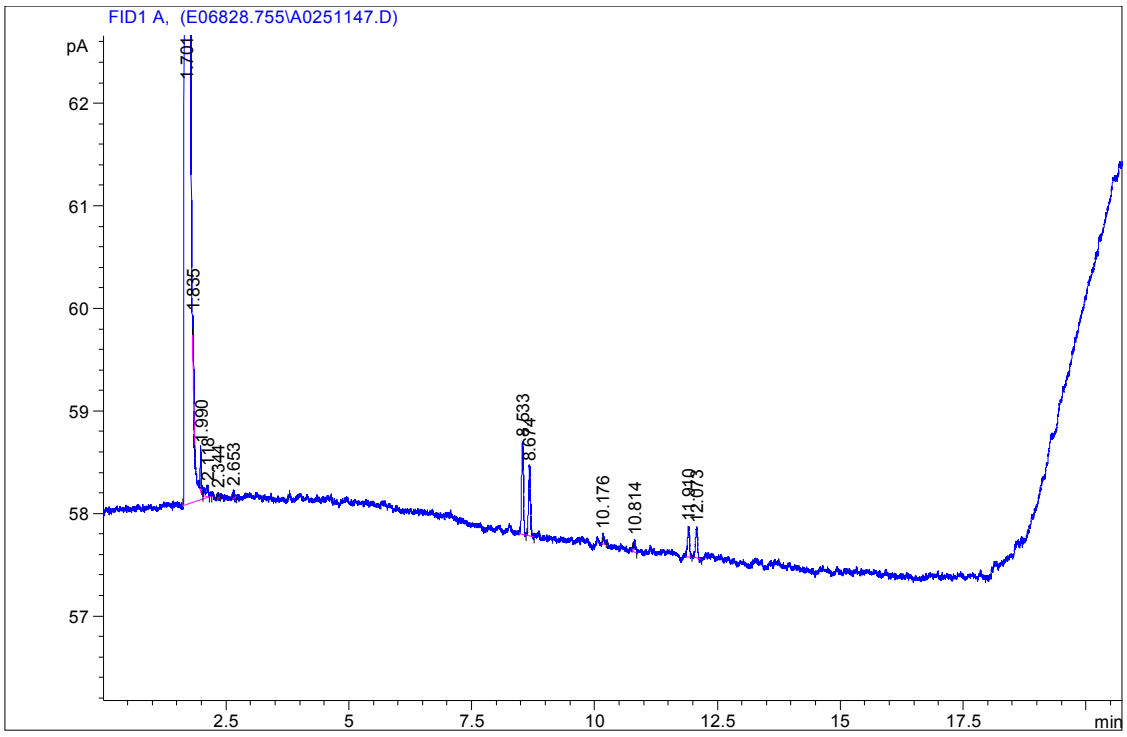

Figure 6. Chromatogram of *Bacillus licheniformis*

Discussion

From the above results, it was observed that the variations were not constant and they were found fluctuating. The observed ammonia values ranged from 0.031 to 0.144 mg L^{-1}, nitrite from 0.014 to 0.314 mg L^{-1} and nitrate from 0.793 to 69.821 mg L^{-1}. There

was considerable increase in nitrite and ammonia levels from 1st to final day. This may be due to the activity of the denitrifying bacteria which was sufficient enough to convert nitrite and ammonia developed in the aquarium tank into nitrate from 1st to final day and subsequently the population may be decreased leading to increase in ammonia and nitrite level. But at the same time the nitrate level was showing a steady increase in the concentration and reached a maximum of 69.821 µg L^{-1}.

A recommended maximum acceptable value for nitrate was given as 1500 µmol (Spotte 1979), based on acute toxicity tests in adult fish. Different concentrations of nitrite ranging from below 0.2 mg L^{-1} NO_2-N (Blancheton 2000) up to 12 mg L^{-1} NO_2-N (Mazik et al 1991; Chen & Lee 1997) were being targeted as safe levels in aquaculture systems. In the Living Seas seawater aquarium, Florida, there has been a continuous increase of nitrate for the first five years of its operation and reached a maximum value of 9700 µmol (Grugric et al 1998).

The present study revealed that the biofilters installed in the aquarium were working well and keeping the nitrogen load in safer and optimum level which was reflected by the steady increase in the concentration of nitrate. However, necessary steps have to be made to reduce the rate of increase in nitrate level in the aquarium.

The water reaching the storage tank was passed through the bioballs kept in the biofilter and resulted in the maximum count due to the beneficial and denitrifying bacterial load available in the biofilter chamber.

The proportionally higher bacterial counts (8.03×10^5 CFU mL^{-1}) obtained from the diseased aquarium water than the healthy aquarium water (1.16×10^5 CFU mL^{-1}) indicated the probable reason for the outbreak of disease. The minimum bacterial counts obtained from the UV sterilized water showed the maximum efficiency of the UV sterilizer. Rajeswari et al (1998) reported significant reduction in bacterial counts in seawater after serial slow and sand filtration, UV treatment and again slow sand filtration for use in hatchery.

The proportionately low count of bacteria could be the result of filtering systems that were provided in aquaria. Rajeswari et al (1999) also suggested that the UV treatment of seawater may be the ideal way to use in marine aquaria for better health management of aquarium fishes.

Three bacterial species *Vibrio parahaemolyticus, Bacillus subtilis,* and *Bacillus licheniformis* were identified by MIS. Species such as *B. subtilis* and *B. licheniformis* occur naturally in fresh and seawater environments, in the intestinal tracts of fishes, prawns and are considered as probiotics for shrimp aquaculture (Moriarty 1998; Moriarty 1999). Bisasiola (1987) reported pop eye disease in ornamental freshwater fishes and stated difficulty in diagnosing the cause of the eye disease and he has pointed out that pop eye condition may be associated with various infectious diseases. Oestmann (1987) attributed management failure as probably the biggest cause of the disease. Rajeswari et al (1999) isolated three different bacterial species *A. salmonicida, V. anguillarum* and *V. parahaemolyticus* from different fishes affected by pop eye disease and attributed the etiological agent to the genus *Vibrio*. Similarly in the present study the pop eye disease appeared to be of bacterial origin and etiological agent may be attributed to the species *V. parahaemolyticus.* The present findings show that marine aquarium water in the recycling system contains significant number of a wide variety of bacteria. Position of various filtration systems in the present aquarium clearly shows that the experimental tank contains nearly pure water without any bacterial cells. The bacteria were introduced into the water from uneaten feed or diets, dead bodies, wounded fishes and excreta of fish and these paths differed based on the species. For example the fish species *P. imperator* and *C. collare* were underfeeding in the initial phases and results in increases of uneaten feed within the tank. It directly influenced the bacterial population which in turn may affect the health of the fish. The food ingested microbial composition has been reported through the estimation of intestinal micro-flora by various authors (Tanasomwang & Muroga 1988; Blanch et al 1999). The presence of high concentration of *Vibrio* in the tanks 3, 4, 5 also resulted in lesser survival rate of the fishes maintained in these tanks. The influence of the associated bacteria, *V. anguillarum* in water, food and initial stages of fish on the survival of fish in captivity has been demonstrated by

Westerdahl et al (1994). Blanch et al (2001) also monitored the bacteria of the aquarium water, particularly the *Vibrio* populations and determined a higher composition of *Vibrio* populations from several exhibition aquaria with a shared water supply. *P. aeruginosa, V. cholerae, V. parahaemolyticus* isolated in the present samples is able to survive and multiply in the gut, mucus and tissues of fish and they are also potential pathogens of man (Janssen 1970; Trust & Money 1972; Weistreich & Lechtman 1973). In recirculating aquaria microbial populations multiplies day by day. This directly affects the health of the fish that are maintained in high concentration of harmful microorganisms. Since bacteria are present in an aquarium and they could also readily be transferred to humans, besides affecting the fishes the presence of potential pathogens in the aquarium could become a hazard to human health.

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