STUDY OF MICROFLORA PATHOGENIC TO FISH IN BIOREMEDIATED SEWAGE EFFLUENT

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ABSTRACT

To assess the suitability of bioremediated sewage effluent from Shehzad Town, Islamabad for fish cultivation, it was tested for the presence of microflora pathogenic to fish and also for its water quality, at National Agriculture Research Centre (NARC), Islamabad. There was no significant difference found in the microflora of fresh and bioremediated sewage effluent and no evidence of disease causing species was observed during the study. A total of 15 fungal isolates and 7 bacterial isolates were obtained from bioremediated sewage effluent. Identified isolated microflora was found to be non-pathogenic to fish. Most prominent fungal species were that of *Aspergillus*, *Alternaria*, and *Penicillium*, whereas *Pseudomonas*, *Proteus*, and *Elizabeth* were prominent bacterial species in both fresh and bioremediated sewage effluent.

KEYWORDS: bioremdiated sewage effluent, microflora

INTRODUCTION

Freshwater available in the form of useable water is present in very low quantity, consequently leading to problems like water scarcity. Water in lakes, ponds, and rivers is only about 0.01% of the total water present in the hydrosphere. With the increase in population, the needs of food and natural resources is also increasing, so to cope with such changing conditions, many plans and interventions are made in the society, which always are not proven fruitful and cause a serious threat to water bodies as well as natural environment. In most of the cases, domestic sewage water is discharged in the form of either, untreated, treated or partially treated into the nearby water bodies, where it results to cause serious sanitary issues and certain other health concerned problems. So its proper treatment is essential to be done before its disposal.

Bioremediation is a pollution control technology that is known to use biological systems to catalyze the transformation or degradation of many toxic chemicals to the one that is less harmful. Waste recovery imply improvement of a component of waste material for its use in a different manner which was the way in its initial stage or it can also be said that re-use denote multiple uses of a given product, and recycling or treating consists of the process of recovering from the waste or pollutants the matter or said new product, which can be re-used or re-introduced in the environment for usage in the same manner or different (Ademoroti, 1996). Recovery, recycling or reuse helps to overcome the problems of waste disposal and scarcity and threat of natural resources.

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Wastewater comprises of an extreme complex of mixture of organic and inorganic materials as well as a complex of ecosystem, consisting of bacteria, fungi, viruses, protozoa, and nematodes (Williams & Baun, 2003). A substance or water body is considered as pollutant when it is perceived to have an adverse effect on the environment and is believed to cause harm to human health. The sources of microorganisms are variable that causes infectious diseases. Therefore, proper treatment of water is necessary and it should also be monitored for the presence of pathogenic microorganisms. Wastewater generated from urban and rural areas after domestic use is a large source of water. It mainly comprises of 99.9% water with some suspended and dissolved organic and inorganic solids, in a relatively very small concentrations (Mara & Cairncross, 1989; UN Department of Technical Cooperation for Development, 1985).

Pathogenicity or virulence is the ability of a pathogen to cause any disease and the degree of pathogenicity in turn depends on the factors such as the ability of the pathogens to colonize a host, or to interfere and cause any disturbance in the defense system of the host, production of toxins, chemical nature of cell components etc. certain factors including temperature, pH, quality of water, and metals present in environment are also believed to be playing a role in pathogenicity.

Fish are susceptible to a wide variety of pathogens present in water, which include both bacterial and fungal pathogens. Many of these pathogens which are capable of causing diseases transform into pathogens when fishes are under physiological imbalanced conditions, nutritionally deficient, or under other stress conditions like; poor water quality, overstocking, etc. the poor quality of water, fluctuation in water temperature, inappropriate temperature, high density of fish, mishandling of fish etc. Because of the reason that fish is in intimate association with aquatic environment, 'water quality' is the most important and foremost factor to be considered for healthy production of fish.

The identification of microbes involves comparison of an unknown micro-organism with the one that has been identified before and is with the similar properties and characters. In the similar way, in this study, the unknown microbes will be identified that are present in the bioremediated water, and also their pathogenicity will be determined, which they are expected to pose to fish. The microbial or micro-floral load will determine the quality of water, as well as the health hazards that may be caused to fish.

The reuse of water in agriculture and aquaculture should be carefully monitored, as the quality of crop and fish produced in such water depends completely on the quality of water. Presence of certain microbes can identify the quality of water and expected health and quality of fish. Indeed, majority of microbes present in treated water are beneficial to fish health instead of causing any harm. So in the present study, it has been evaluated that either this water is suitable for fish production or not. The micro-flora present in water is directly related to the health effects of fish, if this water is being used for fish production.

The present study is conducted at National Agriculture Research Council, Islamabad for analysis of microflora present in bioremediated sewage effluent and also aims to identify the pathogenic microbes present in bioremediated water, which can affect healthy fish production in this water. Freshwater fish and freshwater was studied for the presence of normal flora, to be compared with that one of bioremediated.

MATERIALS AND METHODS

Site Selection

The present study was conducted at the fisheries and Aquaculture Program, Department of National Agricultural Research Centre (NARC), PARC (Pakistan Agricultural Research Council) - Islamabad in order to study the normal flora present in fish and the micro-flora which can be indicated as pathogenic to fish, present in the bioremediated sewage effluent from Chak Shehzad.

Water Quality Analysis

Dissolved oxygen, oxygen saturation, and oxygen pressure parameters were determined by using oxygen meter (Oxi Lab) using standard protocols and also by Winkler's titration method. EC and TDS were determined following the procedure of Richard (1954) by using Electrical Conductivity (EC) meter (water proof TDS tester, dual range). To measure the pH, the micrometer ph meter (jenway 3510) was used. Total alkalinity was estimated by methyl orange indicator (A.P.H.A 1971) following

titration method. BOD was measured by using Winkler titration method.

Microbiological Analysis:

Media Preparation

Merck's Tryptic soy agar (TSA) was used for the growth and study of bacteria present in the water samples. Merck's Potato Dextrose agar (PDA) was used for the growth and study of fungi present in water samples. Prepared media plates were placed in the incubator in an inverted position, for the whole night.

Culturing of Water Samples

For the bacterial culturing, tenfold sample dilutions (10^{-1} to 10^{-5}) were made with sterile saline physiological saline (0.85% w/v NaCl). Aliquots of 0.1 mL of the serial dilutions were inoculated onto the tryptic soy agar plates (TSA) using the spread plate method (APHA, 1998). Cultured plates were placed in incubator set on temperature of 37°C for 24 hours, 100 µL of the appropriate water sample was cultured on to the potato dextrose agar plate (PDA) using the spread plate method. Cultured plates were placed in incubator at 28°C-30°C for 7 days.

Isolation of Microflora

Prepared plates were observed after 24 hours for bacterial growth and morphological characters including shape, size, color, and elevation of the bacterial colonies. Total number of colonies was recorded by taking total bacterial load. Bacterial colonies with same morphological characters were counted separately and three to five representatives of each colony type was streaked on fresh media plates. To obtain pure cultures, each bacterial colony was streaked and re-streaked on fresh media plates. The purified bacterial colonies were stored at 4°C on TSA slopes and were re-subcultured on TSA after every 2nd week. Prepared plates were observed every next day for fungal growth, and observations were made for the color, texture, and size of the growing fungal colonies. The total number of colonies grown on each media plate was counted and recorded. Each grown colony was then carefully picked and cultured on individual PDA plates, for purification. Purified cultures were then stored in refrigerator for identification.

Identification of Microflora

Bacterial strains were identified on the morphological as well as chemical bases. Bacterial slides were prepared for the study of morphological characters. Slides were observed for the characters like, shape, size, outer wall, and staining to differentiate as gram positive and gram negative bacteria. For biochemical identification tests were performed using the criteria provided in *Bergey's Manual of Systematic Bacteriology* and API 20E (BioMerieu, France) methods. Fungal identification was done by slides preparation from the purified cultures. Prior to slide preparation, color of grown colony, its texture and features were observed, which helped to identify the specie of related genera. Compendium of soil fungi was used for fungal identification and study.

Pathogenisity Test

Two trials were conducted for indication of pathogenic status in fish of selected bacterial and fungal species. Six aquariums were used, three for fungal trials and three for bacterial, with six fish (three *Tilapia*, three common carp) in each aquarium. Three fungal species (*Aspergillus niger*, *Aspergillus flavus*, and *Alterneria alternata*) and three bacterial species (*Stenotrophomonas maltophilia*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*) were selected for pathogenesis trials on fish. Dilutions were made of each bacterial and fungal species to study fish behavior under stress. Dilutions of each fungal and bacterial species were prepared in 2 mL of distilled water and this prepared dilution was mixed in each aquarium tagged with the name of the species used for stress. Fish were observed daily for its behavior, continuously for 10 days, to notice any effect on health.

RESULTS & DISCUSSIONS

The effects of water quality are very obvious on the health of fish. When treated water is aimed to be used for fish production, then micro-flora plays an important role in determining the quality of water. Data for physicochemical parameters of water samples taken from the fresh and bioremediated water ponds are shown in Table 1. No significant variation was observed in the parameters studied, among the fresh and bioremediated water ponds. This can be said due to the fact that the results obtained were under the per-

Table 1. Physicochemical analysis of freshwater and bioremediated sewage effluent

Parameters	May	June	July	NEQs (2000)*
Freshwater				
Alkalinity (mEq/L)	49	47	91	50-350
Hardness (kg/mm²)	300	89	90	100-300
pH	8	7.87	8.10	6-9
TDS (mg/L)	370	390	360	360-500
Temperature (°C)	18.5	20.4	20.2	40
Salinity (mg/L)	0.2	0.3	0.3	0.5
DO (mg/L)	9	9	3	3-6
EC (µS/cm)	485	503	490	100-2,000
Bioremediated Sewage Effluent				
Alkalinity (mEq/L)	75	56	155	50-500
Hardness (kg/mm²)	350	151	209	100-300
pH	8	8.21	8.80	6-9
TDS (mg/L)	640	710	590	3,5
Temperature (°C)	18.2	20.4	20.2	12-30
Salinity (mg/L)	0.2	0.3	0.3	< 0.5
DO (mg/L)	3	4.3	5.4	3-6
EC (µS/cm)	501	960	650	150-2,500

Parameters studied: Comparison in experimental months of May, June, and July

* National Environmental Quality Standards. The Gazette of Pakistan, Extra, August 10, 2000

missible limits as proposed by international standards. Water temperature is one of the most influencing environmental factors affecting pond dynamics and both the metabolism and growth of fish (Weatherley & Gill, 1983; Herzing & Winkler, 1986; Boyd, 1990). Water temperature in fish ponds is related to solar radiation and air temperature. In the present study, water temperature was favorable for fish culture as mentioned by Boyd (1990) in both fresh and bioremediated sewage effluent. Water temperature during the sampling period ranged from 18°C to 20°C for both fresh and bioremediated water. Dissolved oxygen varied from 3 to 9 mg/L for freshwater and 3 to 5.5 mg/L for bioremediated water, the recorded dissolved oxygen i.e. 3-5 mg/L in the pond of bioremediated sewage effluent was favorable for fish culture (Boyd, 1990) and comparable with results of Abdel-Tawwab *et al.* (2007) and Ali (2007). Total dissolved solids ranged from 360 to 390 mg/L for freshwater and 590 to 710 mg/L for bioremediated water, pH range was from 7 to 8.80 for both fresh and bioremediated water, alkalinity in freshwater was 47 to 91 in freshwater and 56 to 155 in bioremediated water, whereas total hardness of water was from 80 to 300 mg/L in freshwater and 150 to 350 mg/L in bioremediated water. Fish exposed to soft water are more susceptible to other water quality conditions also. Very hard water can also be a problem. Ideal ranges of water hardness could be 50 to 300 mg/L CaCO3 for optimum growth of fish (Wetzel, 2001). In this study water hardness was found under the permissible limits advised by Pakistan Environmental Protection Agency (PEPA, 1993). Electrical conductivity varied from 485 to 500 in freshwater and 500 to 960 in bioremediated water. The average values describe the difference more significantly.

Taxonomic Composition of Fungal Flora

Fungal isolates recovered from fresh and bioremediated ponds water was identified to species level. Their percentage distribution is shown in Table 2 and figure 1. In total 15

Table 2. Percentage distribution of fungal microflora in freshwater and bioremediated sewage effluent

Months		*A. niger*	*A. flavus*	*A. alternata*	*A. longipes*	*P. expansum*	*C. affinis*	*B. cinerea*	*M. hiemalis*	*R. oryzae*	*T. viride*	*F. roseum*
May	Freshwater	27.91	21.66	37.91	0	12.5	0	0	0	0	0	0
	Biowater	3.03	17.6	16.75	0	13.43	10.16	8.33	0	0	22.33	0
June	Freshwater	11.1	24.06	23.13	0	14.8	0	18.5	0	0	0	0
	Biowater	21.56	7.13	22.1	0	5.12	7.69	12.26	0	9.5	0	14.44
July	Freshwater	22.7	6.03	28.76	0	0	0	0	6.03	4.16	15.53	0
	Biowater	13.7	3.7	6.66	14.8	6.66	9.23	0	9.23	10.16	0	9.23
Mean ± SE*		16.6±1.62	13.36±1.37	22.55±2.37	2.46±0.65	8.75±1.55	4.51±1.33	6.51±0.88	2.54±0.67	3.97±0.67	6.31±1.31	3.94±0.67

Note: Full names of fungal species shown in graph and table

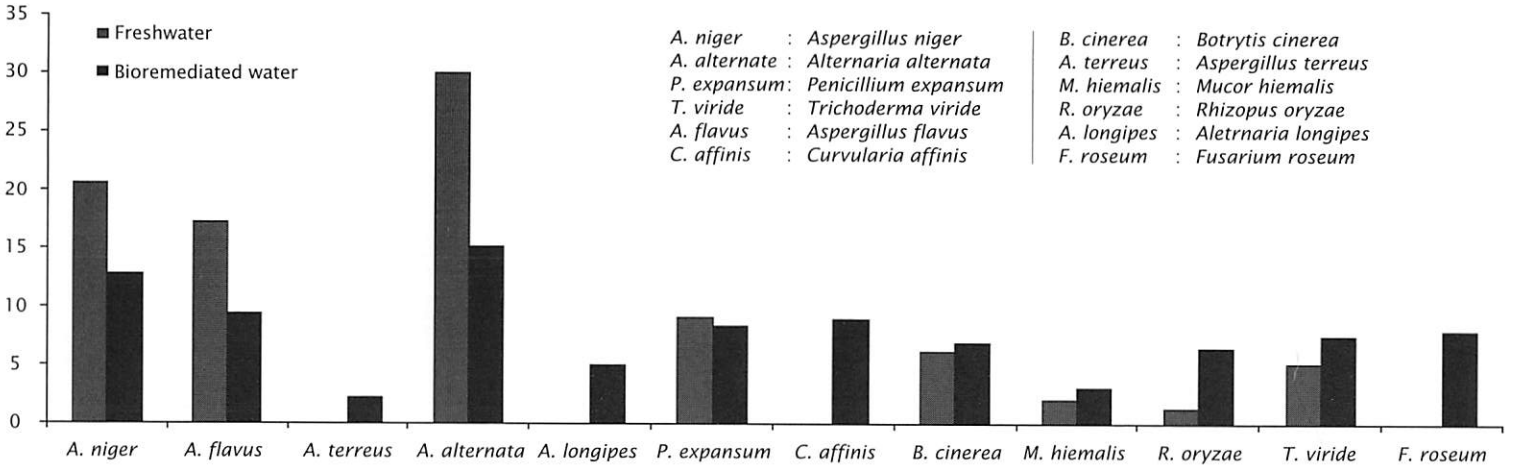

Figure 1. Graph showing percentage distribution of fungal microflora in fresh and bioremediated sewage effluent

fungal species were identified from both fresh and bioremediated ponds water. In freshwater, 10 fungal species were present i.e., *Aspergillus flavus*, *Aspergillus niger*, *Alternaria alternata*, *Curvularia lunata*, *Penicillium expansum*, *Botrytis cinerea*, *Cladosporium cladosporioides*, *Rhizopus oryzae*, *Trichoderma viride*, and *Mucor hiemalis* were present with *Alternaria alternata* being most dominant one. In bioremediated ponds water, 15 species were identified, *Aspergillusflavus*, *Aspergillus niger*, *Fusarium roseum*, *Curvularia lunata*, *Penicillium expansum*, *Botrytis cinerea*, *Mucor hiemalis*, *Rhizopus oryzae*, *Trichoderm aviride*, *Cladosporium cladosporioides*, *Alternaria longipes*, *Aspergillus terreus*, *Cochliobolus spicifer*, *Curvularia affinis*, and *Alternaria alternate* being most dominant.

According to a study, fungal flora of fish were studied and found *Curvularia lunata*, various species of *Aspergillus* and one species of *Cladsporium* from fish body and reported them as the normal flora. From these findings it was noticed that these fungal flora are the part of normal-healthy, partially dead, and diseased fish body (Ravindran, 2001). Therefore, the fungal species found like *Aspergillus falvus*, *aspergillusniger*, *curvularia lunata*, and *cladopsorium*, can be said as the normal flora of fish, which is present naturally on fish body. This normal flora can cause disease in fish under some unfavorable conditions or stress on fish. Fish carry load of micro-flora despite of fact that from wherever it is caught or kept in which ever type of water, as fish comprises of normal flora, which is present naturally on fish. This micro-flora varies depending upon the geographical areas, season, and habitat of fish (Roberts, 1990).

Taxonomic Composition of the Bacterial Flora

Bacterial isolates recovered from fresh and bioremediated ponds water was identified to species level. Their percentage distribution is shown in Table 3 and figure 2. In total 9 bacterial genera were identified from both fresh and bioremediated ponds water. In all populations, 7 bacterial species i.e., *Burkholderia cepacia*, *Elizabethkingia meningoseptica*, *Proteus mirabilis*, *Pseudomonas luteola*, *Stenotrophomonas maltophilia*, *Pseudomonas oryzihabitans*, and *Pseudomonas aeruginosa* were present with *Proteus mirabilis* being the most dominant in freshwater and *Pseudomonas oryzihabitans* being most dominant in bioremediated ponds water. These species were found to be non-pathogenic to fish. Non-pathogenic strains of bacteria were found in both fresh and bioremediated sewage effluent (Table 4 & 5). The found bacterial species were also non-virulent to fish and are not known to cause disease in fish. *Pseudomonas luteola*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Burkholderia cepacia*, and *Elizabethkingia meningoseptica* were the identified species in the present study. Literature and the previous studies revealed that none of this bacterial flora is pathogenic to fish. Huss (1988) reported that when water is polluted with the sewage waste, it may contain pathogenic bacterial species of *Salmonella*, *Aeromonas hydrophilia*, *Shigella*, *Vibrio cholera*, and the hepititus-A virus. According to a study, *Pseudomonas* and *Aeromonas* species are the most dominant isolates among other species in sewage water (Ogbondeminu*et et al.*, 1991).

Table 3. Percentage distribution of bacterial microflora in freshwater and bioremediated sewage effluent

Note: Full names of bacterial species shown in graph and table

Months		*B. cepacia*	*E. meningoseptica*	*P. mirabili*	*P. oryziabitans*	*P. aeruginosa*	*S. maltophili*	*P. luteola*
May	Freshwater	31.6	37.16	31.13	0	0	0	0
	Biowater	0	0	7.76	29.4	22.63	28.70	11.38
June	Freshwater	42.43	6.03	51.43	0	0	0	0
	Biowater	0	28.08	0	31.45	25.70	0	14.68
July	Freshwater	13.86	28.86	53.16	0	0	0	0
	Biowater	0	7.4	0	40.66	9.23	24	0
Mean ± SE*		14.46±1.8	17.92±1.3	23.9±1.7	16.9±3.6	9.5±1.4	8.7±0.6	4.3±1.2

B. cepacia : *Burkholderia cepacia*

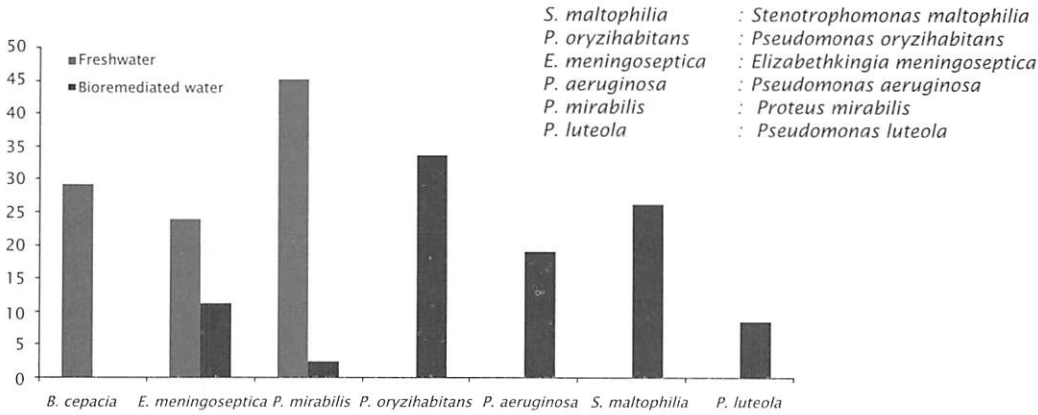

Figure 2. Graph showing percentage distributions of bacterial microflora in fresh and bioremediated sewage effluent

Table 4. Bacterial load in freshwater and bioremediated sewage effluent

	Months					
	May		June		July	
Bacterial load/ mL (CFU/mL)	Fresh water	Bioremediated water	Fresh water	Bioremediated water	Fresh water	Bioremediated water
	4.2×10^2	1.64×10^3	3.2×10^2	5.2×10^2	3.2×10^3	4.4×10^2
	1.6×10^3	1.36×10^3	1.2×10^3	1.2×10^2	2.4×10^4	3.2×10^3
	1.82×10^2	8.4×10^4	3.6×10^2	1.12×10^4	5.6×10^2	7.2×10^2

Table 5. Result of pathogenicity trials

Fungal and bacterial specie for trial	Symptoms of pathogenecity
Aspergillus niger	(-), no symptoms
Alternaria alternata	(-), no symptoms
Aspergillus flavus	(-), no symptoms
Burkholderia cepacia	(-), no symptoms
Elizabethkingia meningoseptica	(-), no symptoms
Proteus mirabilis	(-), no symptoms

The non-pathogenic behavior of identified species was proven to be true in the trials conducted by giving stress to fish by selected fungal and bacterial species, in which fish remained healthy. The identified fungal species in this study are known to be either plant or crop pathogenic or in some cases are human pathogenic, only if they are inhaled directly or in very huge concentrations (Compendium of Soil Fungi, 1980).

CONCLUSION

This experimental study can be concluded as an opportunity to facilitate the use of bioremediated sewage effluent for fish cultivation. Microflora and water quality analysis of bioremediated sewage effluent showed that this water can be utilized for safe and healthy fish cultivation.

RECOMMENDATIONS

Keeping in view, the results of microfloral and water quality analysis, it can be recommended that bioremdiated sewage effluent is safe to use for fish culture. Time to time water quality analysis and proper care can yield even better results.

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