Date 30.12 31.08.97

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. M. S. Islam Trainee Investigator (if any) _____

Application No. 96-026 Supporting Agency (if Non-ICDDR,B) SDC

Title of Study Microbiological investigation of a fish culture farm using wastewater grown duckweed as fish feed.

Project status:
( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population:
   (a) Ill subjects — Yes No NA
   (b) Non-ill subjects — Yes No NA
   (c) Minors or persons under guardianship — Yes No NA
2. Does the study involve:
   (a) Physical risks to the subjects — Yes No NA
   (b) Social Risks — Yes No NA
   (c) Psychological risks to subjects — Yes No NA
   (d) Discomfort to subjects — Yes No NA
   (e) Invasion of privacy — Yes No NA
   (f) Disclosure of information damaging to subject or others — Yes No NA
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) — Yes No NA
   (b) Use of fetal tissue or abortus — Yes No NA
   (c) Use of organs or body fluids — Yes No NA
4. Are subjects clearly informed about:
   (a) Nature and purposes of study — Yes No NA
   (b) Procedures to be followed including alternatives used — Yes No NA
   (c) Physical risks — Yes No NA
   (d) Sensitive questions — Yes No NA
   (e) Benefits to be derived — Yes No NA
   (f) Right to refuse to participate or to withdraw from study — Yes No NA
   (g) Confidential handling of data — Yes No NA
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure — Yes No NA
5. Will signed consent form be required:
   (a) From subjects — Yes No NA
   (b) From parent or guardian (if subjects are minors) — Yes No NA
6. Will precautions be taken to protect anonymity of subjects — Yes No NA
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

I agree to obtain approval of the Ethical Review Committee for any changes affecting the rights and welfare of subjects before making such change.

M. S. Islam
Principal Investigator

_____ Trainee

APPLICATION FOR PROJECT GRANT

1. a) TITLE OF THE PROJECT: Microbiological investigation of a fish culture farm using wastewater grown duckweed as fish-feed.

b) PRINCIPAL INVESTIGATOR: Dr. Md. Sirajul Islam

COINVESTIGATORS:
Dr. M. John Albert
Dr. A. Huq
Mr. Z. Rahim
Dr. Rashidul Huq
Mr. M. Ikramullah
Dr. G. Podder

ADVISORS:
Prof. Rita R. Colwell
Prof. R.B. Sack

c) STARTING DATE: When funds available

d) COMPLETION DATE: 4 years from starting date

e) TOTAL BUDGET REQUIRE: US $ 688,256

d) FUNDING SOURCE:

f) HEAD OF PROGRAMME : Interim Director
Laboratory Sciences Division

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2. AIMS OF PROJECT

a) General aim

Investigation of the microbiological quality of duckweed, the water in which it is grown and the fish to which it is fed. To determine whether there is any health hazard for handlers of wastewater grown duckweed as well as fish fed on contaminated duckweed.

b) Specific aims

1) To study the possible transfer of pathogenic enteric organisms (e.g. viruses, bacteria and parasites) through the duckweed from hostipal based wastewater lagoons to fish ponds.

2) To find out whether fish (fed on duckweed grown in wastewater) harbor pathogenic microorganisms and transmit them to the fish handlers.

3) To monitor the microbiological contamination of fish.

4) To monitor the level of microbial pollution of different wastewater lagoons used to grow duckweed by estimating faecal coliform concentrations.

c) Significance

This study will demonstrate whether duckweed grown in wastewater lagoons is likely to transmit diarrhoeal pathogens among the workers who handle the contaminated duckweed and fish. This study will also determine whether duckweed are useful in improving the microbiological quality of wastewater on which it is grown. This study will help to understand the overall safety of using wastewater grown duckweed in fish culture farms.

3. ETHICAL IMPLICATIONS

Only the environmental samples will be collected and no human subject will be involved.

4. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

a) Background

Duckweeds are tiny, fragile, free-floating, aquatic plants. Their

vegetative reproduction is rapid and they cluster in colonies. forming. a scum across water surfaces that sometimes becomes a minor nuisance in irrigated crops, farm ponds, slow-flowing canals and some hydroelectric facilities.

Duckweeds belong to four genera: *Lemna*. *Spirodella*. *Wolffia* and *Wolffiella*. About 40 species are known. None has distinct stems or leaves but consist of flattened. minute. leaf-like. more or less oval "fronds". a few millimeters in diameter. Many lack roots: white flowers (rare in many species) are so small as to be nearly invisible to the naked eye (Anonymous. 1976).

Reproduction of duckweed is by vegetative means. The daughter fronds bud from reproductive pockets on the side of a mature frond. An individual frond may produce as many as 10 generations of progeny over a period of 10 days to several weeks before dying. Duckweed fronds can double their mass in two days. This is faster than any of the higher plants. Under experimental conditions their production rate can approach an extrapolated yield of 4 metric tons/ha/day of fresh plant biomass.

Duckweeds have long been recognized for their potential as a source of high protein feed for animals. Studies conducted in various countries of the world e.g. USSR. USA. Canada and others have demonstrated the nutritional benefits of duckweed for both livestock and fish. Duckweed-fed animals grew or produced on duckweed-supplemented diets as they did on more traditional diets using either soyameal or fish meal. Mirzapur duckweed project in Bangladesh has demonstrated that duckweeds can be cultivated year round at rates exceeding 700 kg/ha/day. The nutritional value of duckweed is as follows: protein 35-45%. fat 15%. fiber 5-15% and ash 10-15%.

In Mirzapur duckweed project. one hectare of duckweed wastewater treatment plant yields up to one metric ton of fresh plants per day. This daily harvest will produce approximately 100 kg of fish or 100 kg of dried high-protein duckweed meal.

Islam *et al.* (1990a) recently carried out a study on survival of toxigenic *Vibrio cholerae* 01 with a common duckweed. *Lemna minor*. in artificial aquatic ecosystems. They observed that toxigenic *V. cholerae* 01 can survive longer (27 days) on *L. minor* than in water on which the plants were floating or in control water without plants. This study may indicate that the duckweed which grows on the wastewater lagoons may harbour vibrios and other pathogenic bacterial flora. adsorbed from the water. for a long time. When these plants are transferred from the lagoons to the ponds as fish feed. there is the possibility of transferring pathogenic bacteria along with the duckweed. Moreover. workers who are involved in this project may also contaminate themselves during handling of the duckweed and may contract enteric disease.

The fish that eat the contaminated duckweed can also carry pathogenic bacteria on their surfaces as well as in their guts and may also transmit the disease to fish handlers.

Not only duckweed, the zooplankton and phytoplankton which are present in the water system also harbour various pathogenic organisms (Colwell *et al.*, 1980; Huq *et al.*, 1984; Islam *et al.*, 1992a, 1995). These plankton are also eaten by the fish. Therefore, the fish gut can also accumulate pathogenic organisms from the contaminated plankton.

Studies have demonstrated that *V. cholerae* O1 can attach to various zooplankton (Colwell *et al.*, 1980; 1985a Huq *et al.*, 1984; 1990) and phytoplankton (Islam *et al.*, 1989; 1990b; 1995). Not only that other pathogenic organisms e.g., *V. cholerae* non-O1 (Islam *et al.*, 1992a), *Aeromonas* spp. (Islam *et al.*, 1992b), *Plesiomonas shigelloides* (Islam *et al.*, 1991a) can also attach to various phytoplankton in aquatic ecosystems. Therefore, on the basis of all these findings, a thorough investigation needs to be carried out to find out the role of zooplankton and phytoplankton in harbouring various pathogenic microorganisms.

Pathogenic microorganisms are present usually in very low concentrations in environmental samples. To concentrate pathogenic organisms from environmental samples, Immunomagnetic Separation (IMS) technique can be used. Immunomagnetic beads are small super-paramagnetic beads which are coated with specific antibody (monoclonal or polyclonal) corresponding to the surface antigen of target organisms. Test samples are concentrated by centrifugation. Then coated immunomagnetic beads are mixed with the pellet of the test sample. Target pathogens become attached to the coated beads which are concentrated by magnet. Then the beads attached target pathogens are detected by PCR, fluorescent antibody (FA) technique or isolated following standard conventional culture techniques. The IMS technique has been successfully applied to isolate various pathogens such as *Shigella* (Islam and Lindburg, 1992). *V. parahaemolyticus* (Tomoyasu, 1992), *Salmonella* (Kongmuang *et al.*, 1994) etc. Therefore, in the present study attempts will be made to concentrate and detect pathogenic organisms using IMS technique.

Both laboratory and field studies have also demonstrated that *V. cholerae* and other pathogenic organisms can go into nonculturable but viable state in the aquatic environment (Colwell *et al.*, 1985a; 1994). These nonculturable *V. cholerae* can also attach to various zooplankton (Huq *et al.*, 1990) and phytoplankton (Islam *et al.*, 1994). These nonculturable *V. cholerae* in association with various phyto and zooplankton have been detected using fluorescent antibody (FA) and polymerase chain reaction (PCR) techniques. Therefore, studies need to be carried out to detect the nonculturable bacteria from the aquatic environments using both FA and PCR techniques.

Recently, in our laboratory, it has been observed that the PCR reactions are inhibited due to the presence of various inhibitors in the environmental samples. In order to remove the inhibitors, fluorescent antibody cell sorting (FACS) technique could be used. Therefore in the present study FACS machine will also be used to detect enteric pathogens from the environmental samples.

Studies have demonstrated that various enteric viruses e.g., rotavirus (RV), calicivirus, hepatitis virus etc. have been identified in waste water (Williams and Akin, 1986; Deetz *et al.*, 1984; Rao, 1982). These enteric viruses are shed by humans and animals which contaminate the surface water systems in developing countries like Bangladesh. There are reports of viral gastroenteritis outbreaks due to use of contaminated water and food (Gill *et al.*, 1983; Hung *et al.*, 1984). It has been reported from India that virus concentration in sewage is upto 10^5 plaque forming units (PFU) per liter. However, there is no report from Bangladesh regarding viral contamination of sewage and surface water. It was observed that only one infectious RV particle can produce clinical disease (Ward *et al.*, 1986). Enteric viruses have also been detected from shellfish (Portnoy *et al.*, 1975). As enteric viruses can be transmitted from contaminated surface water, we will carry out an investigation in this project to detect RV in the surface water to find out the role of contaminated water as possible sources of viral gastroenteritis.

Some parasites, like other microorganisms, can infect human intestinal tract to cause diarrhoea and dysentery. Among parasites, *Entamoeba histolytica, Giardia lamblia, Ascaris lumbricoides* and *Trichuris trichuria* infections are very common in Bangladesh (Muttalib *et al.*, 1976). Wastewater is an important source of parasites and other pathogens. In developing countries, wastewater treatment facilities are limited. Wastewater is usually discharged directly into various surface water systems and becomes the source of contamination. Surface water is used by more than 80% people of Bangladesh for various purposes. Humans acquire infection with the parasites by ingesting water and food contaminated with eggs or cysts of parasites. Therefore, parasitic infection is very common among people of low socio-economic group who cannot maintain personal, domestic and food hygiene.

Several water-borne outbreaks of *Giardia* and *Cryptosporidium* infections have been reported from the developed countries (D'Antonio *et al.*, 1985). However, there is no environmental study in Bangladesh about the transmission of the parasites from the aquatic environmental sources as well as from the wastewater. Therefore, in the present study, attempts will be made to find out the role of wastewater grown duckweed based fish culture farm in transmitting parasitic diseases to the fish as well as handlers of duckweed and fish.

A project on *Lemnaceae*-based wastewater treatment is going on at Mirzapur in Tangail where "Duckweed farming" is being done on agricultural land using either organic fertilizer or wastewater collected from the Kumudini Hospital Complex. The hospital wastewater is "treated" with duckweed by having it grown in a series of wastewater lagoons. Duckweed, when grown in these ponds. convert substantial amount of organic material and/or fertilizer into plant biomass; they convert nutrients and minerals dissolved in the water column into plant tissue. The nutrient removal rate is directly proportional to the growth rate.

When plants are harvested, nutrients and trace minerals are removed from the system and a dynamic nutrient and mineral sink is established. This forms the basis for a highly effective wastewater treatment technology.

The duckweeds which grow in wastewater lagoons are harvested and used as the only source of food for fish. Therefore, duckweed is utilized for two purposes: one for treating wastewater and the other as food for growing fish (edible). This project has a laboratory in Kumudini Hospital for monitoring chemical elements. e.g.,ammonium (NH_4), nitrate (NO_3), sulphate (SO_4), phosphate (P), total suspended solid (TSS), biological oxygen demand (BOD), etc. of water. All these physicochemical parameters are being monitored regularly on a weekly basis for the influent and effluent treated with duckweed. However, there are no data on the microbiological quality of both influent and treated effluent. There are also no data about the transmission of pathogenic organisms from the wastewater lagoons to fish ponds when the duckweeds are transferred from the lagoons to the ponds where it is used as food for fish. Moreover, there are no data on health hazards involved among the workers who are directly involved with this project. Therefore, to answer all the above questions, this present study is proposed.

Supporting Preliminary Data :

In the preliminary study, it was observed that faecal coliform (FC) concentrations in water, duckweed and fish were similar in the samples of both wastewater and non-wastewater areas except raw wastewater. The mean count of FC in raw wastewater was 4.57×10^4/ml which was reduced to $<10^2$/ml after treatment with duckweed (Table-1).

b) Research plan

Environmental sampling. Water, zooplankton, phytoplankton (zooplanktion and phytoplankton will be separated using plankton nets of different mesh sizes), sediment and duckweed samples will be collected from 12 selected spots of the study area.

Fish will also be collected from fish farming ponds to investigate the presence of pathogenic bacteria in their gut. gills and body surfaces.

Control: In Mirzapur duckweed project. there are two sets of ponds where the duckweed is grown. In one set of ponds. the duckweed is grown using artificial fertilizer. These ponds will be used as control ponds. The people who are involved in harvesting the duckweed from these control ponds are different from those people who are involved in harvesting duckweed grown in wastewater ponds. The duckweed from control ponds are transferred as fish-feed in those ponds which are also different from the ponds in which duckweed from wastewater ponds are transferred. Therefore we will have completely two sets of data, one from control ponds (non-wastewater) and the other from experimental ponds (wastewater area).

Design of waste stabilization ponds: The raw waste is collected and pumped into a primary settling tank of 0.2 hectare area (600 cubic meter capacity which lined to prevent seepage). The raw wastewater is held in the facultative primary tank for 3-7 days and then from there the primarily treated wastewater is pumped into a secondary lagoon system, dimension, length 600 meter x 9 meter width and average 1.5 meter depth. Proper design considering the daily flow wastewater, primary treatment for partial reduction of nutrients and suspended solid settlement and then flowing into the secondary system of 16-18 million liters capacity has been built ensuring a retention of 22-26 days. The wastewater at tertiary state is being monitored for various chemicals and then used for irrigation.

Sampling procedure: Surface water (5 cm below the surface) will be collected in presterilized 1000 ml plastic bottles. Plant samples will be collected by hand using sterile disposable gloves and kept in sterile polythene bags. Sediment will be collected by core sampling device. The top layer of the collected core sample will be analysed. All the collected samples will be transported to the laboratory, inside an insulated foam box with ice bags so that the temperature can be maintained at about 4^0C-10^0C. Samples will be processed within 4 hr after collection in Environmental Microbiology Laboratory of ICDDR,B. Dhaka.

Sampling time: Samples will be collected twice a month for three years. A three year study will provide the data needed to obtain statistically valid results and conclusions. One time sampling will be done for consecutive 10 days and weekly for one month to find out whether there is any day to day or weekly variation in pathogen load.

Number of samples: Seven kinds of samples will be collected, e.g. water, phytoplankton, zooplankton, duckweed, sediment and fish. Stool/rectal swabs will be collected from pond workers at 15 day intervals. A total of 7200 (6048 environmental + 1152 stool) samples will be collected per year, including 864 water samples (12 sites x 24 round x 3 yr.), duckweed 864 (3 kinds x 3 ponds x 2 x 12 months), fish 72 (3 types x 2 x 12 months), rectal swabs 384 (16 handlers x 2 x 12 months). All these samples will be processed to isolate and quantify the target organisms following standard technique. As we plan to collect sample every 15 days interval, 26 samples of each kind from each site will be collected in a year. Apart from being logistically feasible, this sample size (26) will permit us to demonstrate 2 log reduction in the count of microbes in each site.

Culture Technique

Treatment of samples: Plant samples will be blended for at least two minutes with an equal volume of phosphate buffered saline (PBS) to give a homogenous mixture. 0.1 ml of the homogenate will be directly plated onto MacConkey, Shigella-Salmonella (SS), Brucella agar, thiosulfate citrate bilesalt sucrose (TCBS) and taurocholate tellurite gelatin agar (TTGA) media following the drop plate technique for isolation of various categories of *E. coli*, *Shigella*, *Salmonella*, vibrios and *Campylobacter* spp. (Koch, 1981; Collins and Lyne, 1984; Islam *et al.*, 1989, 1990).

A portion of the homogenized materials will also be inoculated into enrichment media (alkaline bile peptone and selenite broth) incubated overnight and then plated onto MacConkey, SS, TCBS and TTGA media (Monsur, 1961) for isolation of vibrios and *Salmonella*. Total and faecal coliform counts will be carried out by plating the homogenate on MFC agar media following standard techniques.

Water samples:

Total and faecal coliform estimation: One hundred ml of water (100 ml each) will be filtered through 0.45 µm membrane filter papers. This will be done in duplicate. Then the filters will be placed on MFC agar media and incubated at 37°C and 44°C overnight to count the total and faecal coliforms respectively following standard procedures (APHA, 1992). Total viable count (plate count) and acridine orange direct count (AODC) will also be carried out following procedures described by Colwell *et al.* (1985a, b). Other bacterial pathogens of diarrhoeal diseases will be monitored in water as described below.

Tests for pathogens: Fifty ml of water samples will be added to 50 ml double- strength alkaline bile peptone water and selenite broth for enrichment of vibrios and salmonellae respectively. After overnight incubation at 37^{0}C, plating will be done on TCBS.

TTGA, MacConkey and SS agar media.

Vibrio-like organisms (from colony appearance) will be picked and streaked on gelatin agar medium (Monsur, 1961) and incubated at 37^{0}C to determine the production of gelatinase and sensitivity to 0/129 vibriostatic compound and then will be inoculated into Kliglers Iron Agar (KIA) and Motility Indole Urea (MIU) agar. Presumptive vibrio isolates will be checked for lysine and ornithine decarboxylase, arginine dihydrolase and for fermentation of mannitol, sucrose, mannose and arabinose (Huq, 1979) and growth in 0, 3, 8 and 10% NaCl. Serology will be done if indicated. Identification of various members of *Vibrionaceae* will be carried out following the procedures described by Islam *et al.* (1981, 1991a, 1992a, 1992b).

The non-lactose fermenting colonies from MacConkey and SS agar plates will be picked and inoculated into KIA, MIU and Simon's citrate agar for biochemical tests to identify *Shigella* and *Salmonella*. Final identification will be done by serological tests following standard procedures (Kelly *et al.*, 1985; WHO, 1987).

Fish: The intestinal contents and gills of fish will be homogenized with PBS and then enrichment will be done for *Vibrionaceae*, *Shigella* and *Salmonella* following the procedures described before (P.8).

Immunomagnetic detection of pathogenic bacteria from the water:

Super-paramagnetic beads will be coated separately with monoclonal or polyclonal antibody specific for the surface antigen of *Vibrio*, *Shigella* and *Salmonella*. Then the water sample (100-500 ml) will be centrifuged at 10,000 rpm for 10 min. Antibody coated beads will be mixed with the resuspended pellet and then the beads will be concentrated using magnet. Magnet attached beads will be washed with PBS to remove non-specifically attached undesired organisms. Finally, target pathogens attached to paramagnetic beads will be isolated following enrichment culture technique and detected them by PCR using specific primers for each pathogens and FA techniques following standard procedures (Islam *et al.*, 1992, 1993).

Immunomagnetic detection of pathogenic bacteria from the duckweed (DW), gills and intestinal contents of fish: 10 g of DW, gills or intestinal contents of fish in 90 ml of PBS will be homogenized in electrical blender for 10 min. The homogenized material will be transferred to a pre-sterilized 4 oz glass container and allowed to settle the debris for 10 min at room temperature. The supernatant will be separated and centrifuged at 3000 rpm for 10 min and the resuspended pellet will be mixed with antibody coated super-paramagnetic beads. Then rest of the procedures will be the same described above in the water section (P.9).

Use of Fluorescent Antibody Cell Sorting (FACS) Machine for Detection of Pathogenic Bacteria

Environmental samples will be concentrated by centrifugation and the pellets will be washed with PBS. Washed pellets will be incubated with monoclonal antibody (MAb) specific for *Salmonella*, *Shigella*, *Vibrio cholerae* O1 and *V. cholerae* O139 for 30 min at room temperature. Following incubation, samples will be washed with PBS and incubated again with fluorescein isothiocyanade conjugated goat antimouse igG. Then the desired enteric pathogens which will be labelled with FITC conjugated monoclonal or polyclonal antibody will be easily sorted out from rest of the population by using a FACS machine following standard procedure (Goe *et al.*, 1995). Finally the sorted enteric pathogens will be detected using PCR technique. This work will be done in the Department of Microbiology, University of Maryland, USA in collaboration with Dr. Anwarul Huq and Professor Rita R. Colwell.

Fluorescent microscopy:

All the samples will be tested by fluorescence microscopy following standard procedures (Islam *et al.*, 1990b). In brief, the samples will be fixed on a slide and then monoclonal antibody specific for the target organisms will be added and the slides will be incubated for 30 min in moist conditions. After washing, FITC anti-mouse globulin goat serum will be added and incubated for 30 min as before. Finally, after washing and drying, the slides will be mounted under a coverslip using buffered glycerol, pH 8.0-9.0 and examined under a fluorescent microscope. Only *V. cholerae* will be counted by fluorescent antibody technique.

PCR technique:

The lower number of target organism will be detected by polymerase chain reaction technique following procedures described by Islam *et al.* (1993). The homogenized material will be centrifuged at low speed to settle the debris. Then the supernatant will be again centrifuged at 11000 rpm for 5 min and the pellet will be used for the extraction of DNA. The detailed DNA extraction procedure is given below:

DNA preparation: Each pellet will be resuspended in 50 µl of solution containing 50 mM Tris-HCl (pH 8.0), 20% sucrose, 50 mM EDTA and 400 µg/ml lysozyme (Sigma, St. Louis, MO, USA) and will be incubated at 37°C for 30 min. Next, 150 µl of solution containing 50 mM NaCl, 1% sodium dodecyl sulfate (SDS) and 500 µg/ml proteinase K (Bethesda Research Laboratories. Gaithersburg, MD), will be added and the mixture will be incubated at 50°C for 60 min. The DNA will then be precipitated with 2 volumes of absolute ethanol in the presence of 0.3 M sodium acetate (pH 5.2) at -70°C and will be harvested by centrifugation at 15,000 X g for 15 min and will be resuspended in 100 µl of a solution containing 10 mM Tris-HCl and 1 mM EDTA (pH 8.0).

DNA amplification: DNA will be amplified by PCR with 10.0 µl of the extracted DNA with 130 ng each of two primers and Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT) per 25 µl of reaction mixture on a Thermal Cycler (Perkin Elmer- Cetus. Norwalk, CT). The PCR reaction will be done for 35 cycles of 1 min each at 94°C (for denaturation), 1.5 min at 60°C (for annealing of primers to single-stranded DNA), and 0.25 to 1 min each at 72°C (for DNA polymerase-mediated extension). Amplified DNA will be separated electrophoretically on a 0.8% agarose gel and transferred to a nylon membrane (Sigma, St. Louis, MO, USA) by capillary action. The primers which will be used for *V. cholerae, Shigella* and *Salmonella* are as follows:

V. cholerae: (CTX-1:5'-CTCAGACGGGATTTGTTAGGCACG-3' and CTX-2: 3'-GCATTATCCCCGATGTCTCTATCT-5')

Shigella: H8 [5'-GTTCCTTGACCGCCTTTCCGATAC-3'] and H15 [5'-GCCGGTCAGCCACCCTC-3']

Salmonella: [5'-CAGTGGTGTCATATCATTGCC-3'] and [5'-GTAAGAAGGTGCTTATACATCTGC-3']

DNA hybridization: Nylon membrane containing DNA will be prehybridized in prewarmed hybridization buffer (5X SSC,[1 x SSC in 0.15 M NaCl plus 0.015 M sodium citrate], 1.0% SDS, 0.5% bovine serum albumin) for 10 min at 37°C. The filters will be hybridized in the same buffer containing 2.7 µl of a 5 µM stock solution of the alkaline phosphatase labeled probe per 55 cm^2 filter at 37°C for 30 min. The membranes will then be washed once for 10 min in 1% SDS-1X SSC at 37°C, once for 10 min in 1% Triton X 100-1X SSC at 37°C and once for 10 min in 1X SSC at 45°C. Hybridization will be carried out following the procedure of Joblonski *et al.* (1986). The membranes will then be transferred to a solution of 7.5 ml of alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 0.02% sodium azide; pH 8.5) containing 33 µl of nitroblue tetrazolium (Sigma) solution (75 mg/ml in 70% dimethylformamide) and 25 µl of 5-bromo-4- chloro-3-indolyl phosphate (Sigma) solution (50 mg/ml in dimethylformamide). The

colour reaction mixture will be incubated at room temperature for 4 h in the dark. The reaction will be terminated by washing the filters with distilled water.

Virological investigation:

Water samples will be tested for the numbers of RV particles. Ten ml water will be fixed with glutaraldehyde to a final concentration of 2% (v/v) for preparation of electron microscope grids following the procedures described by Wommack *et al.* (1995). This work will be done in the Department of Microbiology, University of Maryland. USA in collaboration with Dr. Anwarul Huq and Professor Rita R. Colwell.

Water will be processed first for separating suspended particles. Viruses will be eluted from particles (Appendix-I). The supernatant will be concentrated by filtration of viruses onto virosorb filters and eluted with beef extract (Appendix-II). The eluates (from filtration and particles) will be pooled and further concentrated by ultracentrifugation (Appendix-II).

Viability and quantitation of RV: In samples found positive for RV by EM, viability and numbers of RVs will be determined by inoculation of concentrated samples onto MA104 cell monolayers as described in Appendix III.

Investigation for parasties:

Detection of cysts and ova of parasites from samples: Water sample (100 - 500 ml) will be centrifuged at 3000 rpm for 10 min. The pellet will be resuspended in small volume of 10% (v/v) formal-saline and the total drops will be calculated. One or two drops of suspension will be taken on a clean glass slide and examined carefully using light microscope at a magnification of x100 for cyst of *Entamoeba histolytica*, *Giardia lamblia* and ova of *Ascaris lumbricoides* and *Trichuris trichuria*. The concentration of cyst and ova will be calculated using the formula described by Hall.(1981).

Detection of cysts and ova of parasites in duckweed, gills and intestinal contents of fish and sediment: 10 g of samples. in 90 ml of normal saline will be homogenized in electrical blender for 1 min. The homogenized material will be transferred in a pre-sterilized 4 oz glass bottle which will be kept at room temperature for 10 min to settle the debris at the bottom. The liquid portion will be transferred carefully and centrifuged for 10 min at 3000 rpm. The supernatant will be discarded and the pellet will be resuspended in small volume of 10% (v/v) formal-saline. The total drops of this suspension will be calculated. One or two drops of suspension will be taken on a clean glass slide and microscopically examined carefully at a magnification of x100 for cyst of *Entamoeba*. The confirmed *Entamoeba* cysts positive samples will be

further processed to differentiate into *E. histolytica* and *E. dispar* by specific ELISA (Haque *et al.*, 1995). *G. lamblia* and ova of *A. lumbricoides* and *T. trichuria*. The concentration of ova and cysts will be determined using the following formula.

$$\text{Eggs, ova, cysts/gm or ml} = \frac{TD/SC \times HE}{\text{Total weight (gm) or volume (100-500 ml)}}$$

Where TD = Total drops after suspension of pellet
SD = Total drops of sample examined
HE = Number of eggs, ova or cysts.

Plan for data analysis:

The efficiency of duckweed to adsorb microorganisms from the contaminated wastewater lagoons will be determined by comparing the counts of target organisms of water and duckweed collected from different lagoons. The isolation of members of vibrios, shigellae and salmonellae will also be correlated with coliform counts. Chi-square and t-test will be used as appropriate for comparison and significance levels.

Attempts will also be made to correlate the isolation of pathogens from the duckweed and fish with the isolation from workers involved in the project. The data will be analysed in statistical analysis system (SAS), dBase will also be included. Data will be entered in SOS data entry package. The data will be processed by Mr. M.A. Malek in Archive Unit of Laboratory Sciences Division (LSD).

Justification for further study:

The preliminary data showed that *V. cholerae* O139 and *Salmonella* group C_1 were isolated once during the one year study period by using conventional cultural technique. As it is very difficult to isolate bacterial pathogens from the environment using conventional culture technique, we may have missed the pathogens though they may be present in those ponds. It is, therefore, needed to use more sensitive techniques to be sure that the pathogens are not there and as such there is no microbiological hazard of using wastewater lagoons for growing duckweed which could be used as fish feed in pisciculture. Therefore, we want to carry out a detailed study using IMS, PCR and fluorescent antibody methods.

In the previous study only the coliforms were investigated. However, in the present study, viral and parasitic diarrhoea causing agents will be investigated in addition to bacterial agent.

5. BUDGET SUMMARY

Budget :

	No.of Person	Monthly Rate	% of effort	1st Yr. 1997/98	2nd Yr. 1998/99	3rd Yr. 1999/2000	4th Yr. 2000/2001	Total US $
Personnel								
Project Director	1	7750	60%	55,800	59,706	63,885	68,356	247,747
Co Investigator(NOC-VIII)	1	193	20%	2,325	2,488	2,662	2,848	10,323
Research Investigator (NOA)	1	634	100%	7,608	8,368	9,204	10,124	35,304
Senior Research Officer (GS-VI)	1	460	100%	5,520	6,072	6,679	7,346	25,617
Research Officer (GS-V)	1	355	100%	4,260	4,686	5,154	5,669	19,769
Senior Research Assistant (GS-IV)	2	440	100%	5,280	5,808	6,388	7,026	24,502
Senior Laboratory Attendant (GS-II)	1	192	100%	2,304	2,465	2,638	2,901	10,308
Consultant				5,000	5,000	5,000	5,000	20,000
Travel								
Local				1,200	1,214	1,229	1,300	4,943
Int. travel				3,200	3,200	3,200	3,200	12,800
Supplies & Materials								
Office Supplies				1,000	1,070	1,145	1,200	4,415
Laboratory Supplies				23,000	23,750	24,000	23,000	93,750
Rent, Communication & Utilities				1,200	1,214	1,229	1,300	4,943
Publications cost				500	500	500	500	2,000
Repair & Maintenance				500	500	500	500	2,000
Interdepartmental								
Miscellaneous				500	535	572	600	2,207
Xeroxing				200	214	229	300	943
Total US $				119,397	126,790	134,214	141,170	526,571
Overhead (31%)				37,013	39,304	41,606	43,762	161,685
Total US $				156,410	166,094	175,820	184,932	688,256

wb5.1.ar/Duck

OK
A 12/2/96

Dhaka, Bangladesh

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8. FLOW CHART

Activities of the project, 4 years

First three months: Setting up laboratory methodology.

Three years: Field sampling.

Six months: Data analysis.

Three months: Report writing.

Tasks of investigators

M.S. Islam M.J. Albert Anwarul Huq Zeaur Rahim Leanne Unicomb Rashidul Huq	Standardization of procedure
M.S. Islam Zeaur Rahim M. Ikramullah Kh. Z. Hasan N.S. Shahid	Field investigation
M.S. Islam M.J. Albert Z. Hasan	Data analysis

Advisors†

Professor Rita R. Colwell
Professor R.B. Sack

†Review meeting about the problems and progress of the project will be held every six-month with the advisors.

Table 1: Mean faecal coliform concentration in various components of wastewater and non-wastewater areas

Sample	FC counts (log_{10} cfu/ml or g)	
	Wastewater	Nonwastewater
Water	1.23	1.21
Duckweed	2.87	2.64
Fish Gill	3.58	3.34
Fish intestine	4.33	4.47

(N.B.: Raw wastewater FC counts = 4.66/ml)

wp5.1.ar/duck

APPENDIX-I

ELUTION OF VIRUSES FROM PARTICLES
(Method -APHA, 1992)

1. 2.5 L of water will be centrifuged at 1250 x g for 20 minutes. The supernatant will be further processed as given in Appendix-II.

2. The sediment will be resuspended in sterile 10% beef extract (buffered with phosphate, pH 7.0) containing penicillin. streptomycin and amphotericin B (pond water contains large quantities of fungi). 40 ml will be used per 250 ml original volume.

3. The beef extract material will be pooled. pH adjusted to 7.0 if necessary and mixed vigorously using a magnetic stirrer.

4. The preparation will be held on ice and sonicated at 100 W for 15 minutes.

5. Eluates will be centrifuged at 1250 x g for 15 minutes at 4°C.

6. Supernatants will be stored at -70^{0}C until further processing and testing is performed.

APPENDIX-II

CONCENTRATION OF VIRUSES FROM WATER BY ADSORPTION TO AND ELUTION FROM MICROPOROUS FILTERS
(according to the method of Keswick *et al.*, 1984)

1. 2.5 L of water will be pumped through virosorb filters.

2. Approximately 200 ml of 3% beef extract, pH 9.5, will be added to filters and held for 5 minutes.

3. Beef extract will be pumped through the filters and collected.

4. The pH will be adjusted to 7.5 with 1 N HCl.

5. Beef extract eluate from Appendix-I will be pooled with eluate from step 4 and ultracentrifuged at 100,000 x g for 90 minutes.

6. The pellets will be resuspended in a total volume of 1 ml in 0.15 M Na_2HPO_4 (pH 7.0) with penicillin, streptomycin and amphotericin B to a final concentration of 500 U penicillin, 500 µg streptomycin and 2 µg amphotericin B per ml.

APPENDIX-III

TESTING FOR VIABILITY AND CONCENTRATION OF GROUP A ROTAVIRUS BY DETECTION OF PEROXIDASE-LABELLED FOCI

1. Step 1 and 2 from Appendix-IV will be followed.

2. Supernatants with and without pre-incubation with anti-rotaviral antisera will be inoculated onto confluent monolayers of MA104 cells in 96-well plates in the presence of 1 µg/ml trypsin type IX (Sigma).

3. Plates will be centrifuged at 1000 x g for 15 mins and 50 µl/well of Dulbeco's modified Eagle medium containing 1 µg/ml trypsin type 1X will be added.

4. Plates will be incubated overnight at 37°C in 5% CO_2 incubator.

5. Monolayers will be fixed with 5% (v/v) formaldehyde and air-dried.

6. Monolayers will be stained with optimally diluted (a) rabbit antirotavirus hyperimmune antisera in PBS, then (b) horseradish peroxidase conjugated anti-rabbit immunoglobulins in PBS.

7. A stain containing 3 amino 9 ethylcarbazole will be added and stained cells will be counted in a light microscope at low power.

APPENDIX-IV

Polymerase chain reaction (PCR) technique for detecting human RV P types (Gentsch *et al.* J Clin Microbiol 1992; 30: 1365-11372)

Two amplification steps will be used: the first uses primer pairs that contain highly conserved sequences of gene 4 of P types S. 4. 6, 9, 69M-like and the second uses a cocktail of P type specific primers yielding a PCR product of each P type of different sizes.

A. FIRST (GENERAL) AMPLIFICATION

1) Double stranded RNA (dsRNA) will be extracted from stool samples using a glass powder preparation (RNAID).

2) 1.5 µl of dsRNA will be added to 3.5 µl of dimethylsulophoxide and will be denatured by heating to 97^0C for 5 mins.