chment 1. Library) Date 25/10/93

E SHEET) ETHICAL REVIEW COMMITTEE, ICDDR,B.

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DHAKA 1212

cipal Investigator Dr. Md. Sirajul Islam Trainee Investigator (if any)

ication No. 93-030 Supporting Agency (if Non-ICDDR,B)

e of Study Microbiological investi- Project status:
ion of aquaweed project in ( ) New Study
irzapur. ( ) Continuation with change
( ) No change (do not fill out rest of form)

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

Source of Population:
(a) Ill subjects (Yes) No
(b) Non-ill subjects (Yes) No
(c) Minors or persons under guardianship (Yes) No

Does the study involve:
(a) Physical risks to the subjects Yes (No)
(b) Social Risks Yes (No)
(c) Psychological risks to subjects Yes (No)
(d) Discomfort to subjects Yes (No)
(e) Invasion of privacy Yes (No)
(f) Disclosure of information damaging to subject or others Yes (No)

Does the study involve:
(a) Use of records, (hospital, medical, death, birth or other) Yes (No)
(b) Use of fetal tissue or abortus Yes (No)
(c) Use of organs or body fluids Yes (No)

Are subjects clearly informed about:
(a) Nature and purposes of study (Yes) No
(b) Procedures to be followed including alternatives used Yes No
(c) Physical risks Yes (No)
(d) Sensitive questions Yes (No)
(e) Benefits to be derived (Yes) No
(f) Right to refuse to participate or to withdraw from study (Yes) No
(g) Confidential handling of data (Yes) No
(h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure NA Yes No

5. Will signed consent form be required:
   (a) From subjects (Yes) No
   (b) From parent or guardian (if subjects are minors) (Yes) No
6. Will precautions be taken to protect anonymity of subjects Yes No
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.

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

M. S. Islam
Principal Investigator

Trainee

A-032055

APPLICATION FOR PROJECT GRANT

1.	PRINCIPAL INVESTIGATOR	:	Dr. Md. Sirajul Islam
	COINVESTIGATORS	:	Dr. M. John Albert Dr. Bilqis Amin Hoque Dr. Nigar S. Shahid Mr. M. Ikramullah
	ADVISOR	:	Prof. R. B. Sack
2.	TITLE OF THE PROJECT	:	Microbiological investigation of a duckweed project in Mirzapur
3.	STARTING DATE	:	When funds available
4.	COMPLETION DATE	:	1 year after starting date
5.	TOTAL BUDGET REQUIRED	:	US$ 21,799
6.	FUNDING SOURCE	:	
7.	HEAD OF PROGRAMME	:	Prof. R. B. Sack Associate Director Laboratory Sciences Division

R. Bradley Sack

8. 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 are any health hazards of growing duckweed on sewage water.

b) Specific aims

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

2) To study the possible transfer of faecal coliforms as well as pathogenic enteric organisms through the duckweed from wastewater lagoons to fish ponds.

3) To find out whether fish (fed on duckweed grown in waste water) harbor pathogenic organisms and transmit them to the fish handlers.

4) To study the incidence of enteric diseases among the employees who are directly involved in handling the duckweed and fish.

5) To monitor the microbiological contamination of fish.

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.

9. ETHICAL IMPLICATIONS

Stool specimens or rectal swabs will be collected from all individuals (water and fish handlers) enrolled in this study. The importance of obtaining these

specimens for the study will be explained to the participants by one of us (Mr. M. Ikramullah) and informed consent will be obtained for the collection of specimens. No other specimens will be obtained from humans.

10. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

a) Background

Duckweed are tiny, fragile, free-floating, aquatic plants. Their vegetative reproduction is rapid and they cluster in colonies, forming across the water surfaces, a scum 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 have distinct stems or leaves but consists of flattened, minute, leaf-like, more or less oval "frondi", 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 soymeal or fish meal. Mirzapur duckweed project 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 hector of duckweed waste water treatment plant will yield 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 (Journey *et al.*).

Islam *et al.* (1990) 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 grow 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 food, 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 in their guts and may also transmit the disease to fish handlers.

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 amounts 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 duckweed 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 is for treating wastewater and the other is as food for growing fish (edible). This project has a laboratory of Kumudini Hospital for monitoring chemical elements, e.g. NH_4, NO_3, SO_4, P, TSS, 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 duckweed are transferred from the lagoons to the ponds where it is used as food for fish. Moreover, there are no data on health risks involved among the workers who are directly involved with this project. Therefore, to answer all the above questions, this present study is proposed.

b) Research plan

Environmental sampling. Water and duckweed samples will be collected from sentinel ponds (5), effluent (1), duckweed growing ponds (3)and the fish ponds (3). Fish will also be collected from fish farming ponds to investigate the presence of pathogenic bacteria in their gut.

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 plastic gloves and kept in sterile plastic bags. 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 $4^{o}C$. Samples will be processed within 4 hr after collection in Environmental Microbiology Laboratory of ICDDR,B; Dhaka.

Sampling time. Samples will be collected once a month for one year.

Number of samples. Four kinds of samples will be collected, e.g. water, duckweed, fish and stool/rectal swabs. A total of 384 samples will be collected for one year, including 144 water samples (12 sites x 12 months), duckweed 108 (3 kinds x 3 ponds x 12 months), fish 36 (3 types x 12 months), rectal swabs 96 (8 handlers x 12 months). All these samples will be processed to isolate bacterial flora that are responsible for causing diarrhoeal diseases following standard procedures.

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, SS, Campay-BAP, TCBS and TTGA media following the spread 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 blended plant 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 0.1 ml of the homogenate on MFC agar media following the spread plate technique.

For isolation of Campylobacter spp., R/S or stool samples will be streaked on Campy-BAP media and incubated in candle jar at 42°C for 48 hr. After incubation, the suspected colonies will be checked for Oxidase and catalase test. Then gram staining will be done. Further identification up to species level will be done following the procedures described by Morris and Patton (1985).

The lower number of shigellae will be detected by polymerase chain reaction technique following procedures described by Islam *et al.* (1993). The homogenized plant 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 1.0 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 was separated electrophoretically on a 0.8% agarose gel and transferred to a nylon membrane (Sigma, St. Louis, MO, USA) by capillary action.

DNA hybridization. Nylon membranes containing DNA will be prehybridized in prewarmed hybridization buffer (5X SSC [1 x SSC is 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 *ipaH* 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 Jobloński *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.

Water samples

Total and faecal coliform estimation. Two 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, 1980). 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 salmonella respectively. After overnight incubation at 37°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°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 as described by Islam *et al.* (1981, 1991, 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 for *Shigella* and *Salmonella*. Final identification will be done by serological tests following standard procedures (Kelly *et al.*, 1985; WHO, 1987).

Fish. The intestines of fish will be homogenized with PBS and then enrichment will be done for vibrionaceae, *Shigella* and *Salmonella* following the procedures as described before.

Stool/Rectal swab. Stool samples or rectal swabs (in Cary-Blair) will be collected from diarrhea and non-diarrhoea cases and plated directly on MacConkey, SS, TCBS and TTGA media. A portion of the stool or rectal swab sample will also be inoculated in enrichment media following the same procedures as described above.

Plan for data analysis

The efficiency of duckweed to adsorb microorganisms from the contaminated wastewater lagoons will be determined by comparing the coliform counts of water and duckweed collected from different lagoons. The isolation of members of Vibrionaceae, *Shigella* and *Salmonella* will also be correlated with coliform counts. Chi-square, *Z*-test, 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. To evaluate the risk factors for diarrhoea among the workers who are involved in wastewater-based duckweed project, the incidence of diarrhoea in groups exposed and non-exposed will be compared using relative risks. Statistical significance of these relative risks will be assessed with standard Chi-square tests or Fisher exact tests where applicable. Confounding variables will be taken into account using logistic regression (Kleinbaum *et al.*, 1982).

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).

c) Bibliography

Anonymous (1976) Making aquatic weeds useful : Some perspectives for developing countries. National Academy of Sciences, Washington, D.C.

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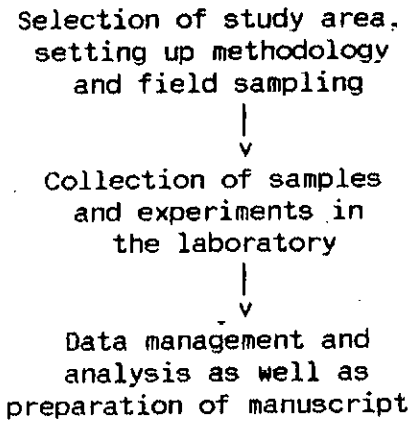
11. FLOW CHART

Activities in 12 months



12. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATORS

Sequence of tasks

Standardization of procedure ------ 2 months
|
v
Testing of specimens ------ 8 months
|
v
Analysis of data and writing reports ------ 2 months

Task of investigators

M.S. Islam M.J. Albert	Standardization of procedure
M.S. Islam M.J. Albert	Setting up PCR technique and screening of samples
M.S. Islam M. Ikramullah N.S. Shahid B.A. Hoque	Field investigation

Detailed Budget

A. Personnel

Designation	Level and Step	Salary per annum	
1. Associate Scientist	NO-C (15%)	US$ 1,700	
2. Senior Research Officer	GS-VI-1 (100%)	6,036	
3. Field Worker (1)	GS-III-1 (100%)	3,432	
			US$ 11,168

B. Operating costs

1) Sampling and processing of water for TC or FC	- 288 @ 7.41	US$ 2,134	
2) Water samples for vibrios *Shigella* and *Salmonella*	- 288 @ 12.70	3,657	
3) Duckweeds for vibrios, *Shigella* and *Salmonella*	- 108 @ 12.70	1,371	
4) Stool samples for vibrios, *Shigella* and *Salmonella*	- 96 @ 12.70	1,219	
5) Transport	-	750	
6) Antiserum/Reagents, non-stock supplies and miscellaneous	-	1,500	
			10,631
Total	-		US$ 21,799

MSI:mh/S10:DUCK.PRT

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH. BANGLADESH
CONSENT FORM

The scientists of the International Centre of Diarrhoeal Disease Research, Bangladesh (ICDDR,B) are studying the transmission of germs of diarrhoeal diseases spread among the workers of the duckweed project. The germs may persist in duckweeds from where they can be ingested and cause diarrhoea. So we will take rectal swab and will examine in the laboratory to see whether or not the rectal swab contains the germs of diarrhoea.

If you or anyone should become sick during the study period, we will either provide treatment for you at home or take you to the hospital.

During this study period and afterwards, your name and any information on illness that you provide will be held confidential. You will not be specifically named or identified in connection with this study. If you choose not to participate, you may still receive treatment at our hospital should you become ill. If you do not agree to participate, you may withdraw from the study at any time. If you are willing to participate in this study, please sign or put your left thumb print below.

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Signature or left thumb print	Date
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Signature of Investigator	Date
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Signature of Witness	Date

সম্মতি পত্র

আন্তর্জাতিক উদারাময় হাসপাতালের বিজ্ঞানীরা ডায়াবেটিস আক্রান্তদের মধ্যে ডায়রিয়ার জীবাণু বিস্তার নিয়ে কিনা সেই বিষয়ে গবেষনা চালাইতেছে। ডায়রিয়ার জীবাণু ডায়াবেটিস এর সাথে লেগে থাকতে পারে এবং সেখান থেকে মানুষের মুখ দিয়ে ঢুকে যেয়ে ডায়রিয়া ঘটাতে পারে। সুতরাং আমরা আপনার লালাগ্রন্থির তুলা লাগানো কাঠি দিয়ে লালার নমুনা সংগ্রহ করব, এবং পরীক্ষাগারে পরীক্ষা করে দেখব তাহাতে ডায়রিয়ার জীবাণু আছে কি না।

গবেষনা চলাকালীন সময়ে যদি আপনি বা আপনার পরিবারের কেহ রোগে আক্রান্ত হয়, তবে আমরা আপনার বাড়ীতে বা হাসপাতালে চিকিৎসার ব্যবস্থা করব। গবেষনা চলাকালীন বা পরবর্তী সময়ে আপনার নাম এবং রোগের যাবতীয় তথ্য গোপন রাখা হবে। গবেষনার রিপোর্টে আপনার নাম প্রকাশ করা হবেনা। আপনি যে কোন সময়ে আপনার নাম প্রত্যাহার করতে পারেন।

যদি আপনি গবেষনায় সহযোগীতা করতে চান তবে নিম্নে স্বাক্ষর বা টিপ সহি করেন।

গবেষকের স্বাক্ষর—

তারিখ:—

স্বাক্ষীর স্বাক্ষর—

তারিখ:—

স্বাক্ষর / টিপ সহি

তারিখ:—