Are waste stabilisation ponds barriers to or reservoirs for cholera?

A Final Report for the Department of International Development

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Summary

This purpose of this proposal was to address strategically important gaps in our knowledge of the fate of toxigenic *V. cholerae* in wastewater and waste stabilization ponds (WSP). In particular, we sought to address the alarming but plausible hypothesis that that WSP might be sources not barriers for the pathogen. A hypothesis based in several reports that the non-toxigenic strains grow in WSP.

We sought to meet this need by attaining the following objectives:

1. To establish, refine and calibrate the methods used for the enumeration of *V. cholerae* exploiting the best available technology.
2. To use the best available methods to determine the presence, numbers and seasonal and diurnal variation in the toxigenic *V. cholerae* in wastewater.
3. An assessment of the occurrence and distribution of toxigenic and non toxigenic *V. cholerae* in WSP in relation to the biological and physico-chemical conditions and the standard faecal indicators.
4. The formulation and dissemination of an assessment of the risk of transmission by WSP effluents meeting the WHO criteria.

We are now able to report that culture based methods for quantifying *V. cholerae* in wastewater and WSP have efficiencies of typically less than 1% and cease to be quantitative at densities below 10^4 innoculated cells/ ml. PCR and dot-blotting failed to improve these methods in our hands. By contrast the fluorescent antibody method gave excellent (almost of 100%) recovery in all milieu, though it was unable to detect low number of pathogens.

All forms of *V. cholerae* were detected in the wastewater and classical seasonal patterns were observed. Nevertheless, toxigenic *V. cholerae* were detected in the wastewater of Dacca City virtually all year round and are thought to be present at high levels ($>10^5$/100ml) at all times. An intriguing 4 week period was observed in the quantities of *V. cholerae* O1.

V. cholerae O1 and O139 were removed (assessed by fluorescent antibody direct counts) from the WSP at a greater rate than the indicator organisms. Similar removals of toxigenic strains were observed when assessed using culture. The non-toxigenic species appeared to proliferate in the WSP. We conclude that the toxigenic and non toxigenic species have different ecologies in WSP and that WSP are a barrier to *V. cholerae* O1 and O139.

The risk of contracting cholera from a WSP receiving a high level of pathogen in the influent and achieving WHO guidelines was estimated. The risk was found to be acceptable in a variety of plausible exposure scenarios. However, the quality of risk assessment will be improved as further epidemiological data (from completed studies) is made available in the peer reviewed literature.

In short WSP are, unequivocally, a barrier to *V. cholerae* O1 and O139 and wastewater reuse using this technology may be promoted with confidence. We believe that this will benefit the poor through improved health and improved livelihoods.

Introduction

The ultimate goal of this study is to prevent the spread of wastewater borne cholera whilst maximising the safe reuse of wastewater for irrigation or aquaculture. The report of this study is, necessarily, technical and academic in tone. We therefore feel that it is important to explain why this is of importance to the poor.

These goals are beneficial to the poor in two ways. Firstly, cholera is, and has always been a disease that disproportionately affects the poor, and in non endemic countries wastewater contamination of food crops is an important transmission route (Swerdlow et al., 1992; Shuval et al., 1985 and Merson et al., 1977). Thus to prevent wastewater borne cholera is to prevent the poor from wastewater borne cholera. Secondly, fears over wastewater borne cholera can persuade public health authorities to either prohibit wastewater reuse altogether or to promulgate onerous, expensive and technically challenging wastewater reuse standards. Yet wastewater reuse in agriculture and aquaculture can be an important source of income for poor peri-urban farmers and fisherman. Thus ensuring that wastewater can be safely reused using simple technologies is a livelihoods issue for the marginal periurban poor. In addition wastewater irrigation can be a very important part of the water resources strategy for an arid area since wastewater is typically the cheapest source of "new water" in a region (World Bank, 1992). Thus promoting a pragmatic, but safe, approach to wastewater reuse can be an important part of ensuring that the cost of water is kept down, which in turn ensures that water supply, with cost recovery, can be extended to as many poor people as possible.

The more narrow purpose of the study was to provide public health authorities with the information that they would need to define policy with respect to wastewater reuse and the spread of cholera. In the remainder of this introduction, we briefly set out the background to such a policy decision and the four objectives of the study in an overview which is a précis of a more wide ranging review (Curtis, 1996).

The species *V. cholerae* can be divided into 3 groups on the basis of the serotype: *V. cholerae* O1 caused the last seven pandemics of the disease, *V. cholerae* O139 which emerged in Bengal in 1992-1993 and, initially supplanted the older serotype and the O1, non O139 *V. cholerae* which are predominantly non pathogenic. The O1 and O139 serotypes carry a toxin (are toxigenic) and this toxin is the basis for their ability to cause the eponymous disease. The toxigenic and non-toxigenic serotypes are very closely related (West et al., 1986) and all forms of the organism are thought to be able to survive, grow and even proliferate in the environment, particularly favouring eutrophic (algal rich) environments and brackish environments.

The quantification of toxigenic *V. cholerae* in wastewaters and effluents is problematic. There are very large discrepancies between microscopic count (organisms that can be seen when identified with a fluorescently labelled monoclonal antibody) and counts based on culture (Huq et al., 1996). The efficiency of traditional and microscopic methods is unknown. It has been hypothesised that the organisms which are visible, but not culturable have entered a hypothetical physiological state in which they are viable but not culturable. All the quantitative work on the fate of *V. cholerae* in wastewater has been undertaken using the culture based methods.
No long term studies of the concentration of toxigenic organisms in wastewater have been published. The short term studies available report concentrations of the

culturable pathogenic forms of the organisms to vary from location to location ranging from 2-10^7 toxigenic organisms/100ml. The non-O1 *V. cholerae* appear to be ubiquitous in wastewater with no reports of a failure to cultivate this organism from wastewater in any of the four inhabited continents (Curtis, 1996).

Conventional wastewater treatment plants are not a barrier to bacterial or viral pathogens unless tertiary disinfection processes are incorporated and, most importantly, routinely operated and maintained.

Waste stabilization ponds (WSP) are able to remove pathogens from wastewater. WSP are comprise a series of basins through which the wastewater is fed. Large algal communities form in the basins or ponds resulting in high pH and high oxygen concentrations combine with other factors to ensure that wastes are stabilised and pathogens are removed. Consequently, WSP are particularly appropriate wherever wastewater irrigation or aquaculture are being considered or wherever downstream users may be exposed to pathogens discharged in treated effluents. WSP are arguably the wastewater treatment system of choice for most poor and middle income communities.

In WSP bacterial and viral pathogen removal is generally assessed using faecal coliforms. Faecal coliforms and faecal streptococci are known decline in WSP at a similar rate to most bacterial and viral pathogens (Oragui et al., 1987). However, it is not clear if *V. cholerae* decline at the same rate as faecal coliforms.

Studies of non O1 *V. cholerae* strongly suggest that these organisms actually grow in WSP (Lesne et al., 1991; Ventura et al., 1992) and that the high pH and algal concentrations that are detrimental to faecal coliforms are beneficial to non-O1s (Lesne et al., 1991). There is evidence that *V. cholerae* O1 is removed from WSP, however, it is not convincing. Kott and Betzer (1972) assessed the removal of cultured seeded organisms from a 70 litre aquarium and reported 1.62 removal. However, little is known about the environmental conditions in the "WSP" or the experimental conditions in general that the study cannot be regarded as in any way authoritative. Oragui et al., (1993) did report that naturally occurring *V. cholerae* O1 El tor declined below an undefined detection limit after a hydraulic retention time of between 4 and 12 days in a short study in pilot scale WSP in Brazil.. The removal rate from the WSP appeared to plateau in the last few ponds in the series studied by Oragui et al., (1993). Whilst this decrease in removal has been attributed to methodological inadequacies (Curtis, 1996) there has been essentially no evidence to prove that this observed decrease in removal did not represent a low level of colonisation in the WSP. There is moreover, absolutely no information on the fate of *V. cholerae* O139 from wastewater treatment systems.

It is unreasonable to ask public health policy makers to support an enlightened wastewater reuse policy whilst there is considerable doubt about the numbers of toxigenic *V. cholerae* in wastewater and their fate in wastewater treatment plants. The reassuring evidence we do have is based on limited studies solely employing culture based methods of unknown, but in all probability inadequate, efficiency.

The purpose of this was to assist the public health policy process by attaining the following objectives:

5. To establish, refine and calibrate the methods used for the enumeration of *V. cholerae* exploiting the best available technology.
6. To use the best available methods to determine the presence, numbers and seasonal and diurnal variation in the toxigenic *V. cholerae* in wastewater.
7. An assessment of the occurrence and distribution of toxigenic and non toxigenic *V. cholerae* in WSP in relation to the biological and physico-chemical conditions and the standard faecal indicators.
8. The formulation and dissemination of an assessment of the risk of transmission by WSP effluents meeting the WHO criteria.

In this report we seek to show that we have achieved all these objectives and that future policy makers will be able use this work to promote wastewater reuse employing WSP with confidence, even in areas of endemic cholera.

The primary route of dissemination is by peer reviewed academic paper. Though this method is slow, it is authoritative. As a consequence each objective is represented by a draft manuscript.

Part 1: Methods Development

Objective 1

"To establish, refine and calibrate the methods used for the enumeration of *V. cholerae* exploiting the best available technology."

This work is to be presented as a platform presentation at the International Water Associations conference on "Water and Health" in Cape Town in September 2003.

A Comparative Study on the Efficiency of Recovery of Toxigenic *Vibrio cholerae* in Waste Water Samples Using Various Laboratory Techniques

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Introduction

There is ample evidence that wastewater has spread cholera in the past, typically when food crops or seafood become contaminated. Wastewater has been strongly linked with outbreaks in South America (Swerdlow *et al.*, 1986). However, the examination of wastewater for *V. cholerae* 01 is more difficult than the examination of the rice water stools of cholera cases (Barui *et al.* 1972; Gerichter *et al.* 1972; Finkelstein 1973; Barua and Burrows, 1974). The classical method, based on enrichment and selective media, is efficient during an epidemic but may fail at its very beginning, when only small numbers of cholera vibrios are present in sewage water. It has been shown that the possibility of detecting small numbers of cholera vibrios in heavily polluted substrates depends not only on the number of cholera vibrios in the sample examined, but also on the general bacterial load as determined by standard plate count (Sechter *et al.* 1975).

In sewage water with a low bacterial load (10^5 to 10^6/ml), the *V. cholerae* may be isolated even if the number goes <10 organisms/ml. However, in sewage water with increased bacterial load, there must be an increase in number of cholera vibrios in the specimen e.g., if the total bacterial load is 10^8/ml, at least 10^2 to 10^3 *V. cholerae*/ml should be in the sample. Such high numbers of toxigenic *V. cholerae* are not expected to be present in sewage water at the beginning of an epidemic. Increasing the volume of sample examined does not substantially improve the result because it mainly depends on the ratio between the number of toxigenic cholera vibrios and the number of other microorganisms in the sample (Sechter *et al.* 1975). This situation is further complicated when *V. cholerae* non-01, in huge number, is present in a sewage sample with very low number of *V. cholerae* 01 and 0139. Moreover, the possible contribution to disease transmission by non-culturable forms of the organism (Roszak and Colwell, 1987, Barer *et al.* 1993) poses another problem for detection. Since non-culturable cells cannot be detected by culture methods, we applied culture independent method to detect *V. cholerae* in parallel with growth-based methods.

An ability to anticipate the appearance of cholera in a population would have important public health implications, allowing optimal allocation of resources for both prevention and treatment of disease. In countries where cholera exhibits a seasonal behaviour characterized by fluctuations in incidence, environmental surveillance can play an important role in cholera control. In 1974, a cholera surveillance program in a South Africa mining community isolated *V. cholerae* 01 from sewage lines before the report of first clinical case of cholera (Isaacson *et al.* 1974). Madico *et al.* (1996) found that *V. cholerae* 01 could be identified in sewage in Lima before occurrence of disease in the community. According to Franco *et al.* (1997), in sites with heavy sewage contamination, *V. cholerae* could still be detected before the onset of cases in the community in a viable but nonculturable (VBNC) form. The VBNC forms, as detected by direct immunofluorescence, may have counts that exceed viable counts by several order of magnitude and may be present at times when no *V. cholerae* can be detected with standard culture techniques (Huq *et al.* 1990). In an ecological study of *V. cholerae* in Vellore, Jesudason *et al.* (2000) demonstrated that although no cultivable 01 and 0139 strains were detected in the environmental sources, flourescent antibody staining detected dormant forms of both serogroups.

Waste stabilisation ponds (WSP) are regarded as the technology of choice for wastewater treatment in developing countries (Feachem *et al.* 1983). Numerous studies of stabilization pond systems in different climates have shown that this process is one of

the most efficient at reducing levels of faecal indicator bacteria and pathogens such as Salmonella spp. in sewage water (Walker *et al.* 1977; Baleux and Troussellier 1983; Legendre *et al.* 1984; Curtis, 1987, 1991; Lesne *et al.* 1991; Boussaid *et al.* 1991). Little research has been reported on the behaviour of opportunistic pathogen bacteria such as toxigenic *V. cholerae* in sewage treatment systems. It is clear that *V. cholerae* and *E. coli* did not behave in the same way in water subjected to stabilization pond treatment. *V. cholerae* favour algal rich environments and high pH values; precisely those conditions associated with the rapid decline of *E. coli* in WSP. A simple search for faecal pollution indicators to evaluate the effluent water's bacteriological quality would not always adequately indicate the health risk represented by the presence of *V. cholerae* in these environments (Mezrioui *et al.* 1995).

Four studies report on the occurrence of *V. cholerae* in WSP (Kott and Betzer, 1972; Lesne *et al.*, 1991; Ventura *et al.* 1992; Oragui *et al.* 1993). The fullest of these,(Lesne *et al.*, 1991) studied non 01 *V. cholerae* and faecal indicator organisms (*V. cholerae* 01 was not detectable). They found that the numbers of *V. cholerae* were negatively correlated with those of faecal indicators and positively correlated with algal concentration and temperature. These findings were corroborated by Ventura *et al.* (1992) who reported their work on full-scale systems in Lima, Peru in a letter to the Lancet. Neither of these studies found evidence that WSP were effective in removing *V. cholerae* from wastewater. In contrast, Oragui *et al.* (1993) and Kott & Betzer (1972) found a declining trend on non-01 *V. cholerae* number in experimental WSP. If we are to arrive at a coherent view on the efficacy of WSP in cholera control this confused picture must be reassessed in light of current views on the ecology of *V. cholerae* and minimum engineering and biological standards for characterising the performance of WSP systems.

Polymerase chain reaction (PCR) is a new tool to detect low number of organisms from environmental samples. The application of PCR protocol in sewage samples for predicting cholera outbreaks has not been reported so far. Besides PCR technique, dot blot hybridisation technique was also applied in our present investigation to detect *V. cholerae* 01 and 0139.

The basic objective of the present investigation was to establish, refine and calibrate the methods to be used in enumeration of *V. cholerae* using the best available technique. A critical aspect of this process was to determine the sensitivity of the methods in different sample materials and with the organism in different physiological states. This will allow us to place rational confidence intervals on any estimates of the numbers of target organisms in the different systems investigated. Although there are many reports documenting the occurrence of *V. cholerae* in wastewater and environmental samples, few have defined the sensitivity of their methods. Quantitation of specific cholera serogroups (01 and 0139) against the background of other serogroups and competing microorganisms in samples presents a significant technical challenge, which can only be met by labour intensive methodologies in the first instance. Here, we deployed such established methods (MPN techniques) as a baseline and subsequently enhance their efficiency by combining them with newer techniques. In the present investigation four different methods e.g., culture, FA, PCR and dot blot hybridisation were compared to find out the efficiency of recovery of toxigenic *V. cholereae* 01 and 0139.

MATERIALS AND METHODS:

Fluorescent Antibody method:

Approximately 10^6 cells/ml of *V. cholerae* tox^+ (*V. cholerae* 01 OGET, Lab Sl. 216, a clinical isolate from Bakergonj, Bangladesh) was prepared in standard diluent solutions (Normal saline, 0.85%NaCl) and the inoculum was diluted to 0, 10^{-1}, 10^{-2}, 10^{-3} dilutions to give approximate concentrations of 10^6, 10^5, 10^4, 10^3 cells/ml. The number of cells/ml at each dilution of inoculum was recorded using FA technique. A dummy sample with previously confirmed of having no *V. cholerae* tox^+ cell was used as negative control. Each dilution of *V. cholerae* inoculum and negative controls were mixed with the four sub-samples of WSP water in the ratio of 50:50. Then all the WSP water sub-samples were subjected to FA technique by using the procedures described by Islam *et al.* (1990).

In a comparative study to check the efficiency of FA technique in seeded wastewater the above procedures were repeated for wastewater of settled sewerage (taken after primary sedimentation tanks).

Test samples of wastewater and waste stabilisation pond water were brought from two selected sites: Pagla Waste Water Treatment Plant and Matlab. Samples from each site were brought two times and the whole procedures were repeated.

3-tube MPN method:

A stock of *V. cholerae* (tox^+) cells was prepared at a concentration of 10^5 cells/ml by taking a colony from an 18-hour old plate and suspending it in the appropriate diluent and the dilution was confirmed by standard plate counting procedure. Dilutions of stock was mixed with pond water or wastewater so as to give final cell count of 10^4, $5x10^3$, 10^3, 10^2, 10^1, 10^0,10^{-1},10^{-2} cells/0.1 ml in the test samples (Table 1). Similar volumes of sterile diluent (normal saline) were also mixed with pond water or wastewater to serve as negative controls (Table-1). Appropriate volumes of test and negative control samples were inoculated into 1/10 APW arranged into sufficient range of 3-tube MPN concentrations and then the tubes were incubated at 37°C for overnight. Enriched samples from each tube were sub-cultured onto TTGA plates, incubated at 37°C for overnight and then suspected colonies from each plate were tested with specific antiserum for *V. cholerae* 01 and 0139.
Wastewater and waste stabilisation pond water from Matlab and Pagla were used in the experiment and each test was repeated.

5-tube MPN method screened with culture, PCR and DBH:

A stock of cells containing approximately 10^5 /ml target (tox^+) *V. cholerae* was prepared and subsequently quantified by standard plate count on LB agar, nutrient agar (NA), TTGA and TCBS plates and by direct microscopic count by using FA technique. As this study was undertaken at a realistic range of *V. cholerae* (tox^+) concentrations of 10^2-10^{-2} organisms/ ml i.e. 10^4 to 1 organism/100 ml, the original stock of (tox^+) *V. cholerae* was appropriately diluted to 10^3cells/ml. Then 1 ml, 0.1 ml and 0.01ml of the diluted stock was added into 100 ml of Pagla waste stabilization pond water (test WSP), 100 ml of settled sewage (test SS) and 100 ml of sterile distilled water (positive control). So the ultimate concentrations of *V. cholerae* (tox^+) become 1000 cells/100 ml, 100 cells/ml and 10 cells/100 ml in the inoculated samples. Furthermore, 100-ml of Pagla stabilization pond water (Control WSP) and

settled sewage (control SS) were also taken for detection of pre-existing *V. cholerae* (tox^+) cells. Total plate counts of SS and WSP on NA plate were also enumerated. Each test and control sample was inoculated into alkaline peptone water following 5-tubes MPN method in which the ultimate strength of APW became 1/10X. After overnight incubation at 37°C each tube was then screened for *V. cholerae* (tox^+) cells by sub-culturing the enriched sample on TTGA plates followed by serology. One ml. of enriched sample of each tube was centrifuged at 13,000 rpm for 15 minutes and the DNA was collected from the pellet by heat-extract method. The *ctx* gene of the extracted DNA was amplified by PCR using appropriate primers under the recorded conditions and the PCR product of each sample was further analysed and confirmed by dot blot hybridisation using ECL technique. The whole experiment was repeated using separate samples of SS and WSP of Pagla Wastewater Treatment Plant.

Detection of single seeded organism in APW when mixed with Pagla WSP and Settled Sewage:

Approximately $2x10^5$/ml cells of *Vibrio cholerae* (tox^+) was prepared in diluent solutions and subsequently quantified on LB or nutrient agar and by FA method following standard procedures. The suspension was diluted from 10^{-1} through to 10^{-8} or onwards until end point of direct count was passed by at least one order of magnitude. Each dilution of inoculum was mixed separately with wastewater (test) and sterile diluent (positive control) in the ratio of 50:50. A similar volume of sterile diluent (negative control) was prepared, diluted and mixed similarly. All the samples were inoculated in 1/10 APW and then incubated for overnight at 37°C. Following incubation each enriched sample was subcultured onto TTGA plate and after overnight growth at 37°C suspected colonies of tox^+ *Vibrio cholerae* were screened by slide agglutination test with specific antisera. Then Extraction of DNA from the collected pellets of each enriched sample (1 ml) was done by simple heat extraction procedure. The *ctx* gene of the extracted DNA was amplified by PCR using appropriate primers under the recorded conditions and the PCR product of each sample was further analysed and confirmed by dot blot hybridisation using ECL technique. The experiment was done separately by using settled sewage and waste stabilisation pond water of Pagla and the preparation of each sample was replicated.

PCR Technique:

All the targeted samples were subjected to a polymerase chain reaction (PCR) with two primers (CTX-1: 5′-CTCAGACGGGATTTGTTAGGCACG-3′;
CTX-2 : 3′-GCATTATCCCCGATGTCTCTATCT-5′) corresponding to nucleotide bases 712-735 and 990-1013 of the *ctx* operon of *V. cholerae* 01 (Shirai *et al.*, 1991). 10µl portion of sample was used as the template for PCR. The PCR was performed in a 50µl volume of a final mixture containing 5µl of buffer solution (50 mM KCl, 20mM Tris-HCl, pH 8.4), 1.5µl of $MgCl_2$, 1µl dNTP (0.2 mM of each of dATP,dCTP, dGTP and dTTP), 0.75µl of each primer, 0.25µl of Taq DNA polymerase (2.5U) and 30.75µl of milli-Q water. The reaction mixture thus prepared was then subjected to PCR using an automated DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The conditions used were 5 min. at 95°C for initial denaturation of DNA and 35 cycles, each consisting of 1 min. at 95°C (denaturation), 1 min. 30 sec. at 57°C (annealing), and 1 min. at 72°C (extension), with a final round of extension for 10 min. at 72°C. The amplified fragments were analyzed by agarose gel (1.5%) electrophoresis and then by visualizing under UV light after staining with ethidium bromide. All the positive samples generated the expected 302 base pair fragment of the *ctx*A gene of the cholera toxin (CT) operon of *V. cholerae* (SHIRAI *et*

al., 1991). Positive and negative controls were always taken along with test samples in each batch of PCR.

Dot Blot Hybridisation:

Appropriate sized Hybond™ nitrocellulose membranes were cut and several sites were marked where 10 µl of each sample (PCR product) was dotted in 2µl aliquots. The membrane was then subjected to denaturation solution (0.5M NaOH, 1.5M NaCl) for 5 min. and neutralizing solution (1.5M NaCl, 0.5M Tris-HCl, P^H 7.5) for 1 min. The membrane was then dried, wrapped with Saran Wrap and placed DNA side down on a UV transilluminator for 5 min. for cross linking the DNA to the membrane. Then ECL 3′-oligolabelling, hybridisation, stringency washes and detection were performed following the standard method as shown in the instruction manual supplied by Amersham Life Science, UK (RPN 2130).

RESULTS:

Fluorescent antibody method: The fluorescent antibody method gave relatively good recovery in wastewater and waste stabilisation pond water inoculated at two sites in Bangladesh (figure 1). Box Cox analysis confirmed that the bacteria were almost perfectly log normally distributed and so natural log transformed data was used to regress inoculated versus recovered giving the following relationship:

ln FADTC (recovered –background/ml) = 0.85 + 0.927 (ln inoculated/ml).

The R^2 coefficient was 93.3, the intercept of 0.85 (standard deviation 0.47) was not significantly different from zero ($p = 0.08$) and the slope of 0.927 (standard deviation 0.03) was highly significant ($p<0.000$).

Unfortunately, the overall sensitivity of the method is poor (about 10^4/ml or 10^6/100 ml). Because direct counts exceed plate counts by a factor of at least 10, this implies a "plate count detection limit" of about 10^3/ml.

A complementary nested analysis of variance of the naturally occurring 0139 examined in the controls revealed that there was much more variation between samples (84%) than between sub-samples.

3-tube (MPN) Method: The 3 tube MPN tubes screened with TTGA were evaluated. Box Cox analysis was used and the recommended (natural log) transformation undertaken. The following regression equation was derived:

Vc/100 ml recovered = -1.31 + 1.16 (Vc/100ml innoculated)

The R^2 value was 52% that only half the variation in counts is explicable by the variation in innocula. The intercept (- ln 9.3, standard deviation 2.9) was significantly different from zero ($p=0.007$) and the slope was close to 1 (1.16, standard deviation 0.28). The efficiency of recovery was not correlated with the numbers of organism inoculated ($p=0.683$).

5-tube MPN method screened with culture, PCR and DBH: To refine the methodology and to increase the sensitivity of the protocol we examined the recovery of *V. cholerae* O1 at the low concentrations we expected in wastewater using 5-tube MPN screened with culture, PCR and dot blot hybridisation (DBH). Median recoveries of 1.24% (culture), 1.62 % (PCR) and 0.75 (DBH) were achieved. The % recovery was unrelated to the of the numbers innoculated ($p = 0.186$). The effect of the milieu had a significant impact on the recovery of seeded organisms (Mood Median $p < 0.001$) with median percentage recoveries of 5.3 (WSP), 0.5 (sewage) and 0.0 (distilled water). This effect could not be attributed to the background count which was significantly higher in the sewage (Mood Median $p=0.48$) and the milieu effect remained significant when the background counts were subtracted. The distilled water was introduced to control for osmotic shock effects. However, it would appear this control was too severe perhaps due to the salinity of the wastewater. The impact of osmotic shock on recovery seeding remains difficult to calculate precisely. At low concentrations (10^2-10^4/100ml) there was no significant linear correlation between the numbers of organisms inoculated and the numbers recovered (figure 3).

Detection of single seeded organism in APW when mixed with Pagla WSP and Settled Sewage:

A central assumption of the MPN techniques is that if a single culturable organism is inoculated into a tube, that organism will be recovered. The poor recoveries observed above imply that this is not true we sought to determine the true endpoint for seeded organisms in single tubes. Baseline counts were made using the fluorescent antibody direct count and a variety of agar, TTGA agar gave the highest culturable baselines and was used. The sterile diluent controls had an endpoint of 1 (TTGA base line) and 10 (FA baseline). By comparison the endpoint in seeded wastewater and pond water was between 100 and 100,000 greater than the endpoint in the diluent (figure 4). This is consistent with the poor recovery observed.

A number of tactics have been employed to improve the FA method. Further concentration of samples using cross flow filtration gave disappointing results, the FA count dropping to zero after concentration. This is presumably due to interference from co-concentrated organic matter.

We believe that the anomalous MPN ratios and general poor recovery is at least partly attributable to competition from other organisms especially other vibrios. Polymixin B (25, 50 and 75 U/ml) was used to try and improve the performance of MPN because it is inhibitory to wide range of Vibrionaceae. Polymixin B supplements did not improve recovery.

Direct PCR and dot-blotting from the environment also gave very poor results (not shown) and is unlikely to be effective in environmental samples.

Discussion

A linear correlation between inoculum and recovery of toxigenic *Vibrio cholerae* in settled sewage and waste stabilization pond (WSP) water of Pagla and Matlab (two selected sites in Bangladesh) was found by FA technique (Figure 1). The results suggest excellent recovery (by virtue of a slope of ~ 1) and little misidentification of non-target material (which would have lead to an intercept of > 0). FA technique also

proved better result in the detection of single seeded organism in APW when mixed with Pagla WSP and settled sewage (SS). Figure 4 shows that the FA count always yielded about 1 log higher count than the TTGA count. The supremacy of FA technique in detection of toxigenic *V. cholerae* was also reported from other investigators (Huq *et al.*, 1990; Islam *et al.* 1990, 1994a; Jesudason *et al.* 2000). However, Huq *et al.* (1990) had reported, for the first time, the presence of *V. cholerae* 01 throughout the year by using the FA technique to detect organisms in >63% of plankton samples collected from Matlab, Bangladesh when the organism could only be recovered from less than <1% the samples examined.

The 3-tube MPN method did not produce satisfactory result. In theory, a slope of 1 and an intercept of -9 in conjunction with the low correlation coefficient the imply that 3 tube MPN though approximately internally consistent, has a low efficiency of recovery (<0.1%) which is further compounded by high degree of error. Poor recovery at < ln 9 organism/100 ml (~10^4/100 ml) suggests that this is the approximate detection limit for three tube MPN using culture. This may be insufficient to recover epidemiologically significant concentrations of toxigenic *V. cholerae* in wastewater.

PCR technique is widely used by many microbiologists because of its advantage over other DNA-based detection methods or immunoassays for its simplicity and speed. The detection limit of the PCR of *ctx*A gene of *V. cholerae* 01 was determined by Shirai *et al.* (1991) to be 1 pg of chromosomal DNA or broth culture containing three viable cells. Three sets of primers were initially used in the PCR assay developed by them and the best results were obtained with the first set of primers amplifying 302-bp region. In our present investigation the same primer sequence was used. PCR has been used by various investigators to detect *V. cholerae* toxin genes in food and in pure cultures (Fields *et al.* 1992, Koch *et al.* 1993). Cholera toxin genes were also detected in "viable but non-culturable" *V. cholerae* 01 in laboratory microcosms employing PCR (Hasan *et al.* 1992). In environmental specimens, the concentration of toxigenic *V. cholerae* is usually very low making it difficult to detect them. According to the observations of some groups (Shirai *et al.* 1991; Ramamurthy *et al.* 1993) there exist many inhibitors in stool specimens, which interfere with hybridisation and PCR assays. Varela *et al.* (1994) demonstrated that PCR can be directly applied to stool swabs without any DNA purification steps and this was achieved by simply boiling the samples to liberate the DNA and diluting the stool samples to avoid the inhibition by substances present in the stool samples. This simple boiling step was performed in our experiment for extraction of DNA from sewage and WSP samples for further analysis by PCR and thus our method obviated the cumbersome need for DNA extraction. Dot blot hybridisation with a non-isotopically labelled chemiluminescent oligonucleotide probe confirmed the ctx specificity of the PCR products but did not significantly increase *V. cholerae* detection limit.

In 5-tube MPN screened with culture, PCR and dot blot hybridisation (DBH) the median recoveries were not statistically significantly different (Kruskall Wallis, p= 0.865, n= 30). Recovery by culture method was poor in case of both settled sewage and WSP samples. There was with, one exception, no significant ($p>>0.05$) correlation between methodologies, thus good recovery in one method would not indicate good recovery with another. The exception was PCR and DBH in WSP water, which were significantly ($p> 0.001$) correlated ($R=0.99$). The two detection methodologies were highly ($R = 0.7$) but not significantly ($p = 0.14$) correlated in

sewage. We deduce that although the different methodologies are all poor they are complementary because they vary in different ways. The lowest detection limit for toxigenic *V. cholerae* in SS and WSP samples was ~10^4 cells/100 ml. as determined in this technique (Figure 3).

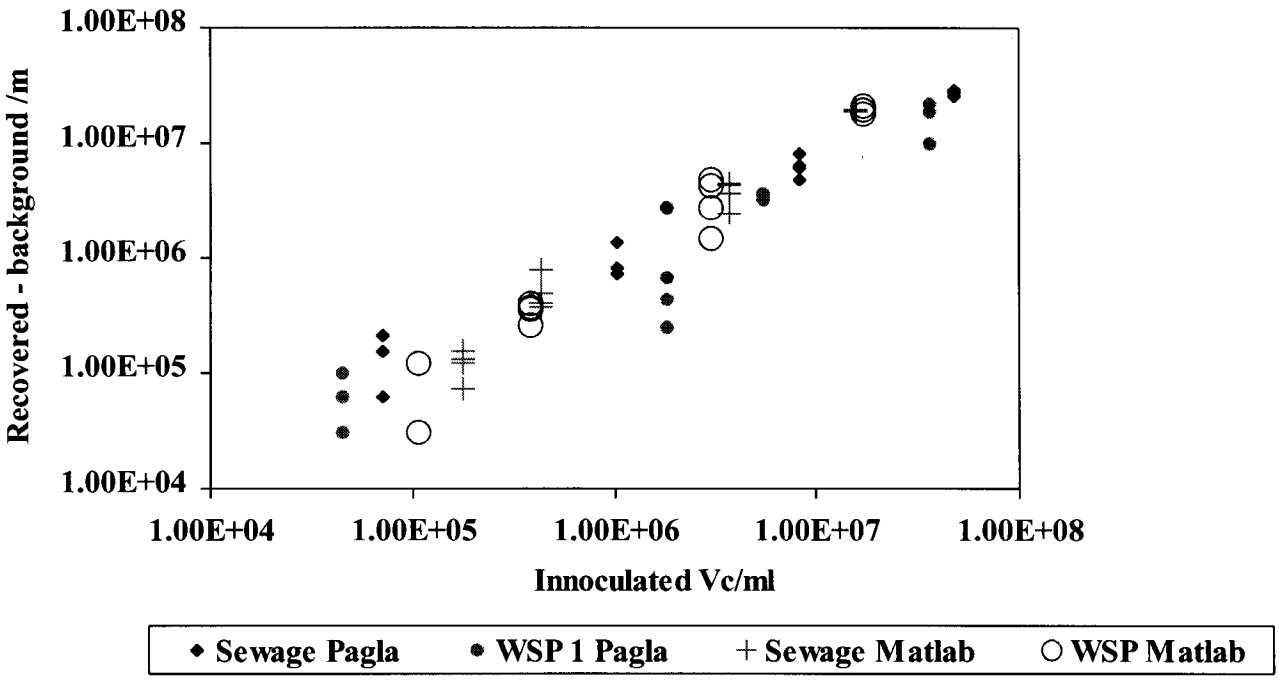
The anomalous MPN ratios suggest that a single organism cannot be detected by the MPN method, thought this is a central assumption of the MPN approach. We therefore tested the following hypothesis: Can any method detect 1 seeded organism in APW when mixed with Pagla WSP water and Settled Sewage? The results showed that it is not possible with the existing techniques (figure 4) and at least more than 50 organisms should be present in culturable form for a positive detection by these methods. From figure 4 it is also evident that endpoint for toxigenic V. cholerae was lower in both culturable and FA count in case of SS samples than that of WSP. This indicates more difficulties in the recovery of toxigenic *V. cholerae* from WSP samples.

This work shows that the best available methodology for quantifying toxigenic *V. cholerae* is poor. In essence MPN methodologies have an efficiency of about 1-0.1% whilst and are very highly variable. Moreover, at low counts MPN appears simply not be quanitative, because there is no relationship between the numbers inoculated and the numbers recovered. By contrast the FA technique gives excellent recoveries ~100%, but has a very low sensitivity about 10^6/100 ml (equivalent to 10^5 culturable bacteria/100 ml) and gives no indication of whether the organism is dead or alive.

Table 1: Different mixture volumes to observe the effect of competitors and inhibitors occurring in pond and wastewater on the recovery of O1/0139 in 1/10 alkaline peptone water by colony identification.

Test sample				Negative control	
Volume O1 (ml)	Volume dilute O1 (1:1000)	Volume pond or waste water (ml)	Approx. Total O1 cells per 0.1 ml	Volume Diluent (ml)	Volume pond or waste water (ml)
1	0	0	10^4	1	0
0	0	1	0	0	1
5	0	5	5×10^3	5	5
1	0	9	10^3	1	9
0.1	0	9.9	10^2	0.1	9.9
0.01	0	9.99	10^1	0.01	9.99
0	1	9	10^0	0	9
0	0.1	9.9	10^{-1}	0	9.9
0	0.01	9.99	10^{-2}	0	9.99

Figure 1 Recovery of seeded toxigenic *V. cholerae* in settled sewage and WSP water in two sites in Bangladesh



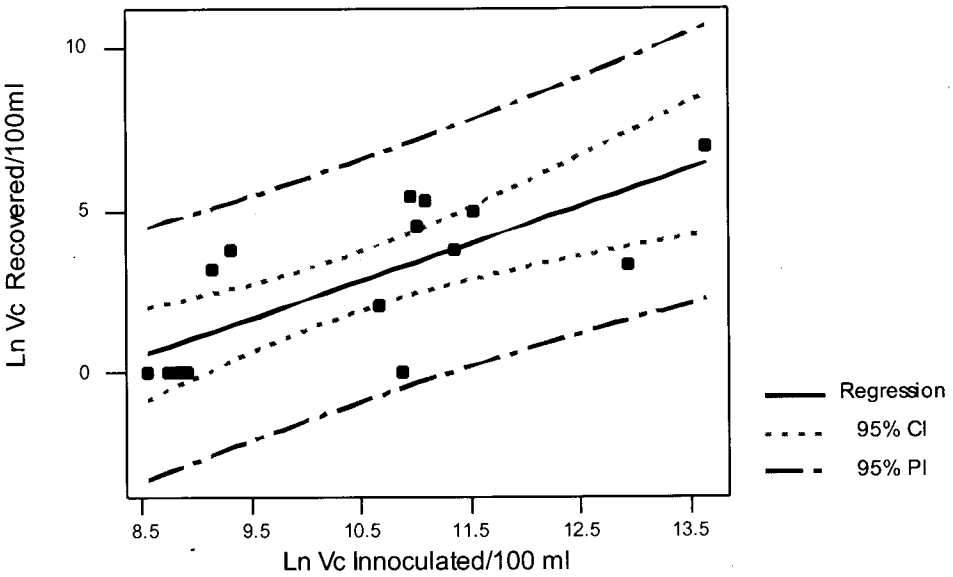


Figure 2. Recovery of Vc in sewage at high concentrations in sewage and WSP water using 3 tube MPN

Figure 3. Recovery of low numbers of toxigenic *V. cholerae* seeded into sewage and pond water using 5 tube MPN screened with culture, PCR, MPN and dot-blot hybridisation.

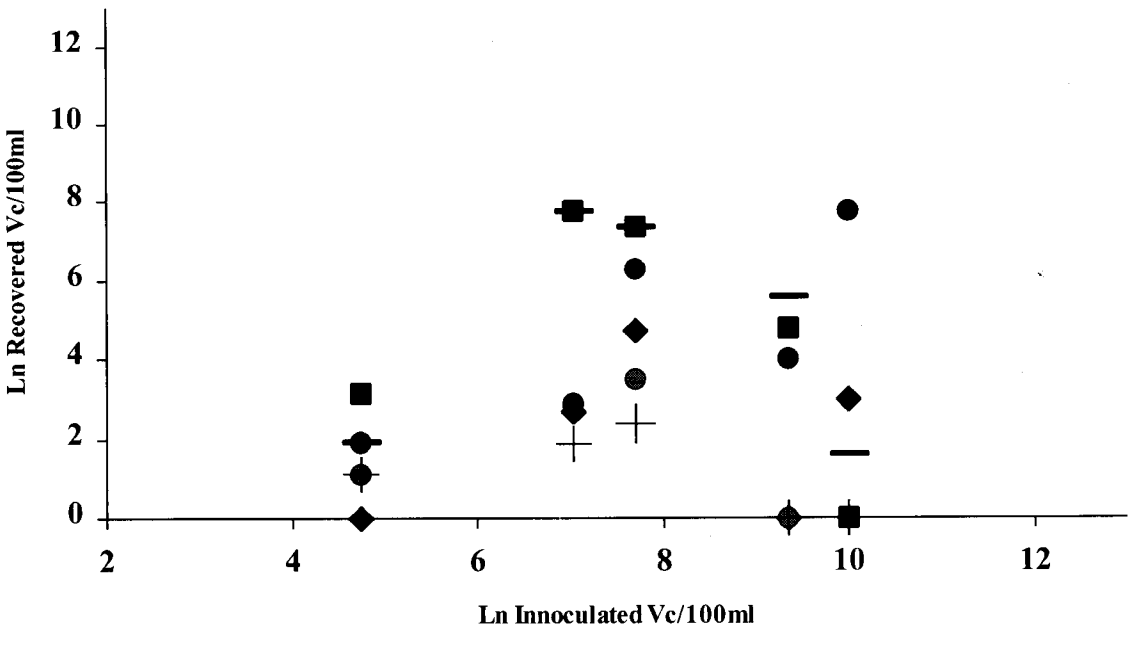
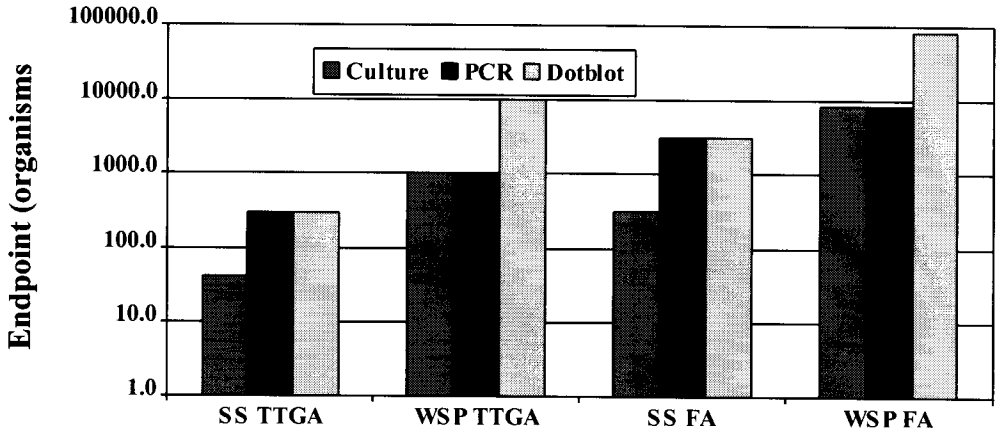


Figure 4. Seeded waters endpoint in 1/10 APW using culture, PCR and dot blot hybridisation. TTGA = agar base line; FA = fluorescent baseline. Each bar is the mean of two runs



Part 2: Cholera Concentrations in Wastewater

Objective 2:

"To use the best available methods to determine the presence, numbers and seasonal and diurnal variation in the toxigenic *V. cholerae* in wastewater."

Cholera concentrations in the domestic wastewater of an endemic city

Introduction

The concentration of toxigenic *V. cholerae* in wastewater is of interest for two reasons. Firstly, it is of direct interest to those trying to remove cholera from the wastewater and secondly, the concentration of cholera in the wastewater may give insights into the dynamics of the disease in the population that uses the sewer. Obviously these two issues are intertwined.

Detailed quantitative studies have of the numbers of *V. cholerae* excreted by carriers and patients have been undertaken, albeit some time ago (Dizon et al., 1966). Those with symptomatic cholera can excrete up to 10^8 comma bacillus/ml of rice water stool, whilst those with asymptomatic infections ("carriers") may shed only 10^5 *V. cholerae*/g of stool (about 10^7 per day). The carriers may be more important there are estimated to be 50 carriers for every person with the disease and those with symptoms of cholera are unlikely to be well enough to use the toilet. The reported numbers of *V. cholerae* are correspondingly variable with 10^7, 10^5, 10^2, and 2 organisms/100 ml being reported in effluents from a cholera hospital, and cities with high, medium and no reported incidence of cholera respectively (Howard et al., 1975; Tamplin and Parodi, 1991; Oragui et al., 1993).

Methodological issues are also a source of variation in the numbers reported in the wastewater. We have established (Islam et al., submitted) that MPN based methods for recovering the organisms from wastewater suffer from very low sensitivities (less than 0.1%), with no quantitative relationship between the numbers of organisms seeded and recovered at low concentrations (10^2-10^4/100ml). Direct microscopic counts by contrast give very good (~100%) recoveries but suffer from inherently low sensitivities. Membrane hybridization methods were regarded as too difficult to apply in practice because of the problematic nature of using P^{32} labelled probes in the field and the unsatisfactory nature of the non radioactive probe labelling protocols available at the time of study. In the light of these difficulties, it was deemed necessary to attempt to assess the concentration of toxigenic *V. cholerae* in wastewater by a number of different methodologies, in the hope that the limitations of the differing methodologies would complement each other.

Finally, raw sewage offers a unique perspective on the dynamics of disease in the community. Normally, disease dynamics are assessed by reports arising from patients seeking medical attention. This not only biases the sampling process (the mildly ill majority will probably not attend) but also introduces a lag in the reporting of the dynamics. The best that the reporting hospital can hope to report is the day the patient sought medical attention, which might be some time after the infection occurred. This inevitably introduces a lag in the difference between the dynamics of the disease and the disease reporting.

Methods

Sampling The wastewater treatment plant in Pagla serves Dacca. The raw sewage entering the plant was sampled at approximately 11.00 each week for 61 weeks. An appropriate volume (2-3 litres) was taken and the total flow (inferred from the hours of operation of the pumps feeding the plant), BOD, pH (both as described in Standard

Methods), mean air temperature of the current and previous week, mean number of hours of bright sunshine in the current and previous week, the mean rainfall for the current and previous week.

The samples were refrigerated and transported promptly to the laboratory in the central Dacca.

Microbiological Analysis The microbiological methods are described in the section describing the methods development. Briefly, the non toxigenic *V. cholerae* were assessed by culture using a standard MPN format, the toxigenic organisms were quantified using: 5 tube MPN screened by examining both subcultures on TTGA plates (MPN culture) or PCR (MPN PCR) or FITC with monoclonal antibodies for O1 or O139.

Statistical treatment of the data. The statistical methods employed are described in a standard text (Sokal and Rohlf, 1996) and were undertaken using Minitab (Minitab, PA, USA).

Results

Analysis and presentation of the data. Immunofluorescence for O1 and O139 and faecal coliforms. The observed distributions for these organisms were not normally distributed (Anderson Darling test, $P << 0.05$) and were consequently examined using the Box Cox methodology. A natural log transformation was selected on this basis because a lambda values of zero was within the 95% confidence intervals for all lambda estimates. The mean values for the natural logarithms of the two organisms were indistinguishable 2298/ml and 2230/ml ($p=0.9$)

MPN data for toxigenic and non toxigenic Vibrio cholerae. The analysis of the MPN data was problematical. A four tube MPN format was employed (1x 50ml; 5 x 10 ml; 5 x 1 ml; 5 x 0.1ml). However, the occurrence of positives at in smaller inoculum volumes were often more frequent than in higher innoculum volumes in all MPN formats (ie MPN screened by culture, MPN screened by PCR and MPN for non-toxigenic strains). Consequently the MPN results were analysed as 4 dilution (50ml, 10 ml, 1 ml and 0.1 ml), 3 dilution (10 ml, 1 ml and 0.1 ml) and two dilution (1 ml and 0.1 ml) MPNs. None of the MPN results were normally distributed (Anderson Darling test, $P << 0.05$) but the large number of zeros observed precluded a Box-Cox analysis. A natural logarithm transformation was employed, it improved but did not completely normalise all the data sets.

Four, three and two dilution MPN were compared for all the MPN formats using: analysis of variance on the natural logarithm transformed data (neglecting non detected results), analysis of variance of the natural logarithm of the data +1 (including the non-detected data) and using the non-parametric Kruskall-Wallis procedure on the raw data. There was a statistically significant difference ($P << 0.05$) between the MPN calculations using different dilutions with all modes of analysis in all MPN formats, except one. The non-parametric test could not distinguish between the different dilutions in the MPN PCR format ($P= 0.225$, median was = 0). In all the ANOVAs the 2 dilution, 5 tube test gave the highest recoveries (Tukey HSD method

family P= 0.05; individual error rate = 0.0193) irrespective of the transformation employed (figure 1). We therefore used the 2 dilution format for all subsequent work.

Statistical Relationships Between Measurements. We examined the extent and statistical significance of the correlation between the various measurements. Statistically significant linear relationships were only observed between the FA O139 and FA O1, FA O139 + O1 and non O1 and the toxigenic *V.cholerae* assessed by MPN culture and faecal coliforms (Table 1). However, even where a significant relationships were observed the correlation coefficients were low. In addition, we also examined a matrix plot of the data to ensure that there were no obvious non-linear relationships between the various measurements, none were observed (not shown).

Time Series A time series of the cholera in wastewater was plotted, in the expectation that we would observe a distinct relationship between the known seasonality of endemic cholera and the levels of the vibrios observed in the wastewater. Though seasonality is evident in the culturable counts, it is less striking in the arguably more reliable fluorescent antibody counts (figure 2). Inter-epidemic periods were marked by a reduction in the numbers of culturable toxigenic organisms and an increase in the negatives in the fluorescent antibody counts. Nevertheless, toxigenic *V. cholerae* were detected in raw wastewater in all but 3 of the 61 sampling events. It is evident that wastewater may pose a hazard virtually all year round and that toxigenic *V. cholerae* are present at high levels ($\sim 10^5$/100 ml) in the absence of high levels of reported disease.

The analysis above is qualitative and we also examined the autocorrelations in the hope of observing some statistically significant periodicity in the data (figure 3). We were surprised to observe a distinct and statistically significant four week period in the data for toxigenic *V. cholerae* by MPN culture and *V. cholerae* O1 assessed by fluorescent antibody counts with autocorrelation coefficients (0.25 and 0.3 respectively). All the toxigenic organisms recovered by culture were from the O1 serotype, thus the culture and FA method were measuring the same organisms (though by completely different techniques). No four week period was observed in the other organisms or by MPN PCR. However, 16 week period was observed the non O1 data and though no single autocorrelation was significant, we were able to show that the autocorrelation coefficients for a 16 week lag were not equal to zero ($p = 0.03$; Leung Box Q coefficient 27.96). A 16 week period was not observed in the other data sets. However, a 32 week lag was observed in the O139 data through a statistically significant autocorrelation (of 0.32). The O139 serotype is relatively rare in Dacca, this lag presumably reflects the classic periodicity in the disease. However no such lag was observed in the other data sets.

We note with interest and curiosity that the observed or suspected periodicity occurs at multiples of four (4, 16 and 32) and attach particular significance to the four week period in the classical serotype because it was observed by 2 independent methodologies. No distinct 4 week lag was observed in the meteorological data or the physico-chemical properties of the wastewater or the volume of the wastewater.

Predictive relationships We sought to find simple linear relationships between meteorological and operational data and the concentrations of the target organisms. No relationship was found between any measure and any of the variables. However a

weak (R^2 14%) but significant effect was found between non O1 numbers and the run time of the pumps which fed water into the plant.

We plan to relate the concentration of toxigenic *V. cholerae* in wastewater to the diagnosis of cholera in the community. However, at the time of writing the necessary data was being sought by ICCDR, B who were constrained by staff availability..

Discussion

The major public health finding in this study is that toxigenic *V. cholerae* is present at all times of the year at significant concentrations ($>10^5$/100 ml) even outside established "cholera seasons". We draw the conclusion of the ubiquity of the pathogen by looking at the results of all the methods, since very often, when one method was negative at least one other method was positive. However, we draw the conclusions about the numbers of organisms by reference to the fluorescent antibody technique. There is a large discrepancy between the concentrations assessed by culture and the concentrations assessed by microscopy. It is thus at logical to consider whether the some of the organisms seen by immuno-fluorescence are capable of growth and infection. However, in our earlier work we have established that recovery efficiencies are so low that they could account for the large discrepancy between culture and microscopy and thus it is best to assume that all the organisms detected are capable of growth and infection.

Though we have not yet been able to compare these findings with the weekly data from ICCDR,B we are confident that the rise and fall in the levels of cholera will be broadly reflected in the rise and fall in the levels of cholera in the city. The However, we suspect that their may be some discrepancies between the two data sets. For example, the O139 serotype appears to present at similar concentration to the O1 serotype, though the latter is thought to be responsible for most of the observed disease in Dacca.

The methodologies for the recovery of toxigenic *V. cholerae* from wastewater are dreadful. Moreover, it is clear from the correlations between the organisms that the week to week dynamics of this group of organisms cannot be monitored by using either their non toxigenic relatives or the faecal coliforms. The only reliable methodology (FA direct counts) is expensive, laborious and quite insensitive. A cheap simple method for quantifying toxigenic *V. cholerae* in wastewater would be a great boon.

Notwithstanding these methodological short comings, we were able to detect some extremely interesting and unusual patterns in the occurrence of toxigenic *V. cholerae* O1 which may have significance beyond the narrow scope of this study. There is intense interest in the relationship between patterns of cholera and environmental variables. As a consequence the observation of a statistically significant 4 week period in the cholera concentrations in the wastewater is of interest. It is important to note that this period was observed in two time series generated using totally different methods. It is therefore unlikely to be a fluke.

It is not yet clear why a four week period is observed. However, we cannot help but notice that some lunar effects will occur on a four week cycle and that there may

therefore be some link with tidal effects. Such an effect might be easily overlooked by studies evaluating cholera dynamic by reference to reports of disease in hospitals. Such reports are typically compiled monthly and therefore incapable of detecting a four week period. Moreover, such reports inevitably relate to the more seriously ill rather than carriers and those with light infections who comprise the majority.

Tables

Table 1. Linear correlation coefficients (upper value) and associated probabilities of significance (lower values) for toxigenic *V. cholerae* measured by MPN screened by culture and PCR, the fluorescent antibody method (for O1 and O139), faecal coliforms and non- toxigenic *V. cholerae*.

	Ln Faecal Coliforms	**FA Ln V.*cholerae* O1**	**FA Ln *V.cholerae* O139**	**MPN culture Ln toxigenic *V.cholerae***	**MPN PCR Ln toxigenic *V.cholerae***	**MPN Culture Ln Non-toxigenic *V.cholerae***
FA Ln V.*cholerae* O1	0.059 0.683					
FA Ln *V.cholerae* O139	0.086 0.555	0.476 0.001				
MPN culture Ln toxigenic *V.cholerae*	0.329 0.031	0.224 0.153	-0.224 0.153			
MPN PCR Ln toxigenic *V.cholerae*	-0.104 0.598	0.179 0.373	0.066 0.755	-0.067 0.740		
MPN culture Ln non-toxigenic *V.cholerae*	0.232 0.077	0.083 0.565	0.187 0.208	0.140 0.376	-0.188 0.349	
FA Ln V.*cholerae* O1+O139	0.233 0.087			0.122 0.442	0.072 0.720	0.336 0.014

Figures

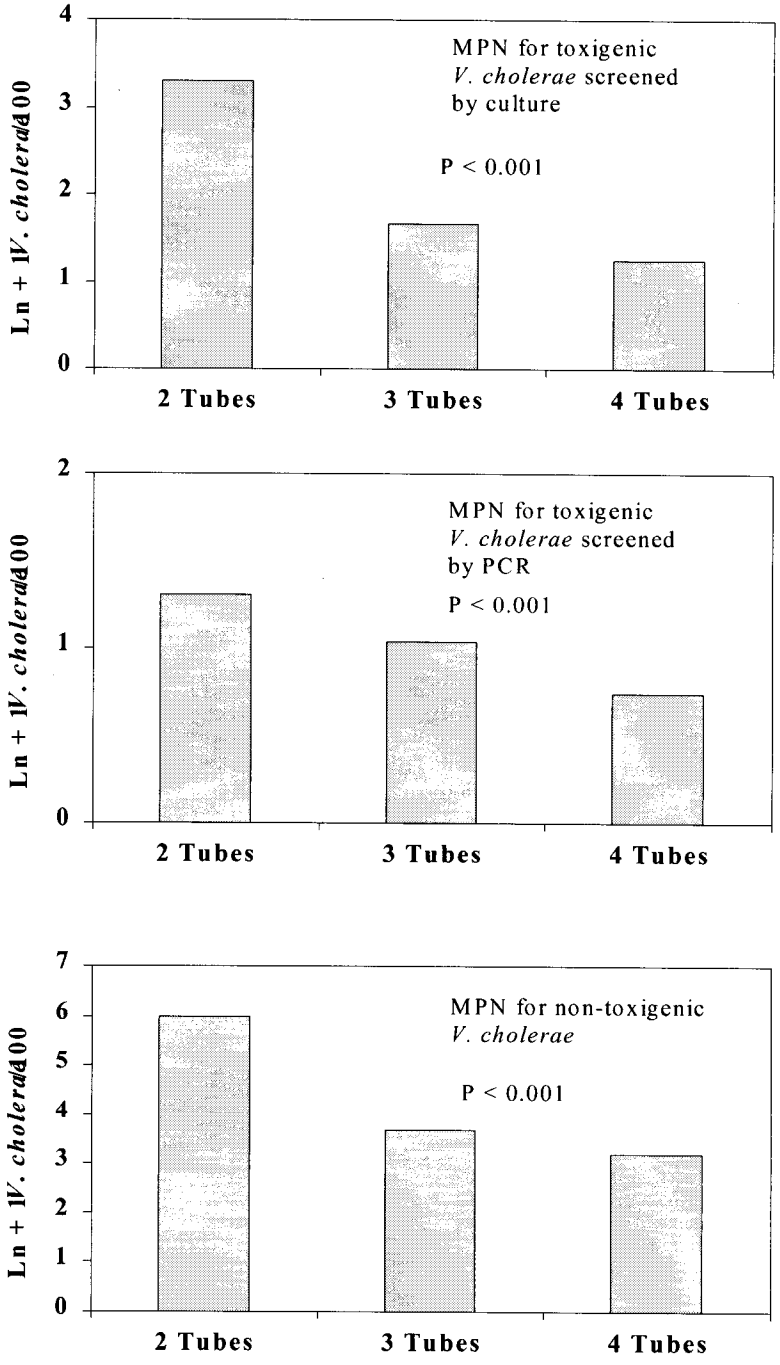


Figure 1. A comparison of the recovery of toxigenic *V. cholerae* by MPN screened by culture and PCR and non toxigenic *V. cholerae* by MPN. The differences were significant (ANOVA, $P<0.001$, $n = 61$) and the recovery by the 2 tube method was greater than the 3 or 4 tube method *V. cholerae* by MPN screened by culture, non toxigenic *V. cholerae* (Tukeys HSD method, family error rate 0.005). Recovery by the 2 tube method was greater than the 4 tube method *V. cholerae* by MPN screened by PCR.

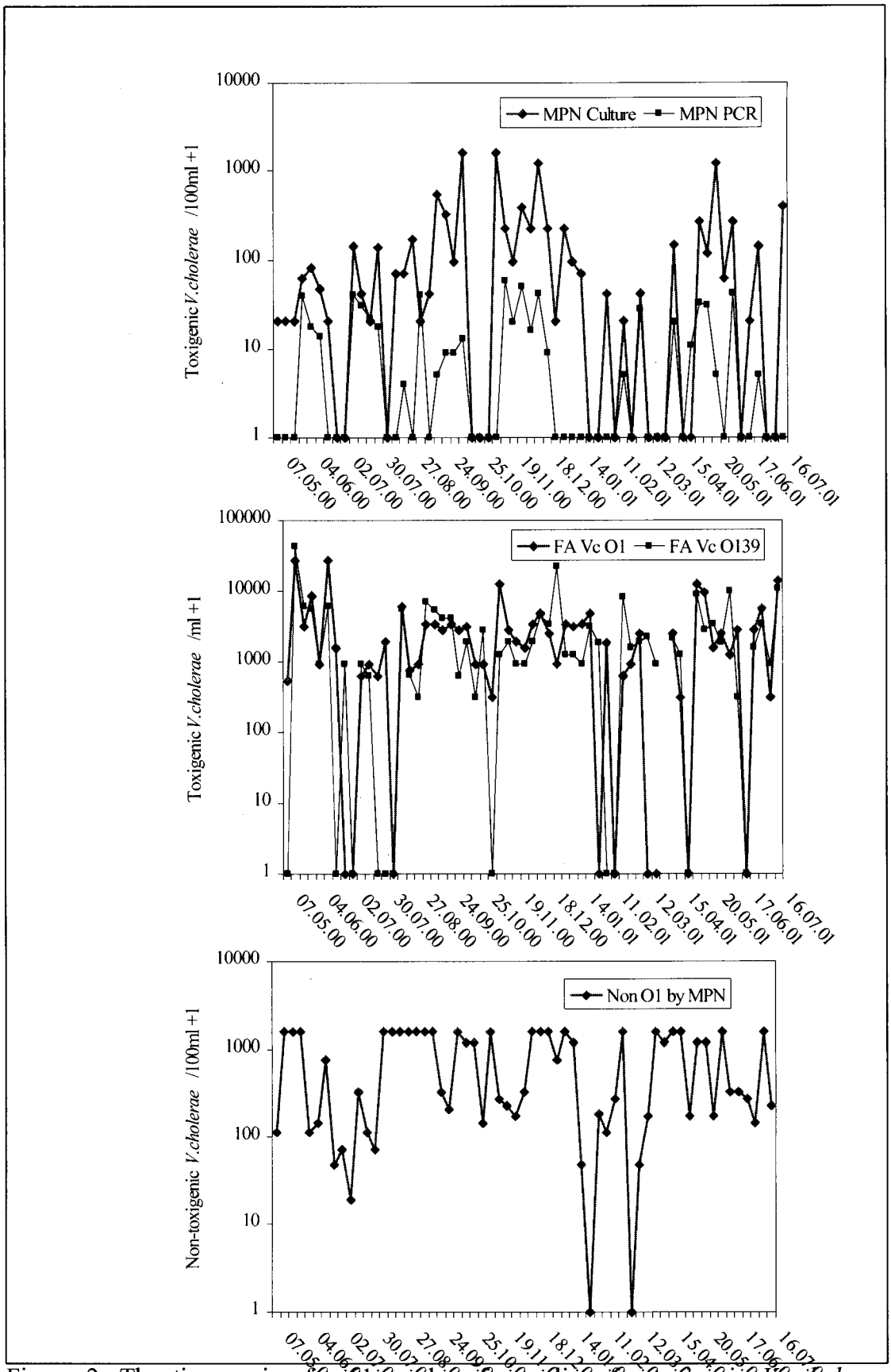


Figure 2. The time series for 61 weeks of sampling for toxigenic *V. cholerae* measured by MPN screened by culture and PCR, the fluorescent antibody method (for O1 and O139), and non- toxigenic *V. cholerae*. A 1 was added to permit the data to be plotted on a log scale.

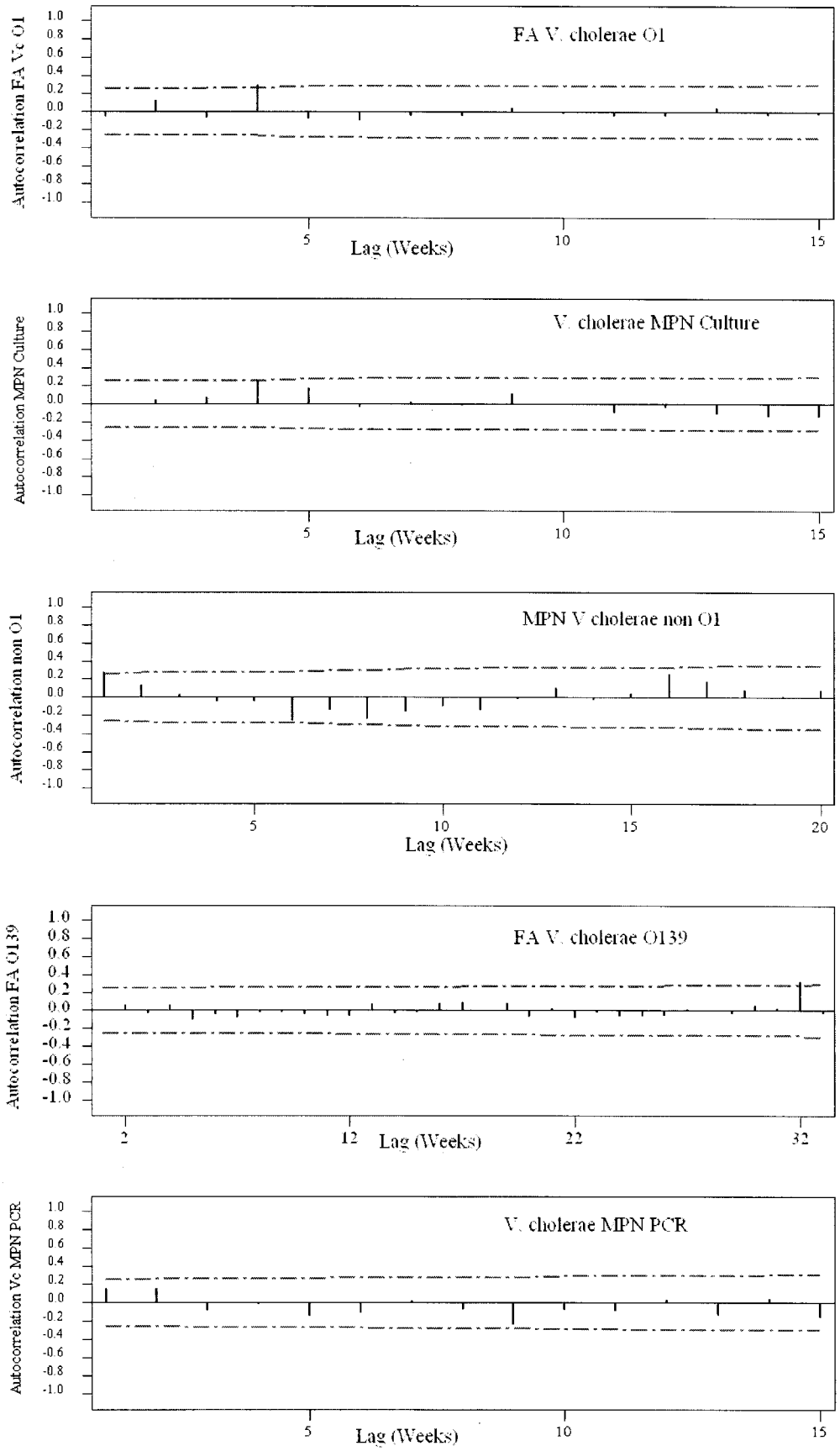


Figure 3. Autocorrelation (bars) with confidence limits (dashed line) and of the counts of toxigenic and non-toxigenic *V. cholerae* in raw sewage in Dacca. Statistically significant autocorrelations were observed at a lag of 4 weeks by FA targeted the O1 serotype and MPN screened by culture.

Part 3: The fate of toxigenic and non-toxigenic *V. cholerae* in wastewater

Objective 3

"An assessment of the occurrence and distribution of toxigenic and non toxigenic *V. cholerae* in WSP in relation to the biological and physico-chemical conditions and the standard faecal indicators".

The fate of non toxigenic and toxigenic *Vibrio cholerae* in a waste stabilisation pond in an endemic region.

Introduction

Waste stabilization ponds (WSP) are a simple, cheap and yet highly effective form of wastewater treatment that are used throughout the world. WSP ideally comprise a series of shallow basins through which the wastewater flows, by gravity, as a complex suite of phenomena contribute to the natural purification of the wastewater. Ecologically, WSP have the appearance of small hyper-eutrophic ponds or lakes, with high algal concentrations (>500 μg/l), high pH (up to 9.5) and high dissolved oxygen concentrations (>20mg/l).

WSP have exceptional pathogen removal properties, especially in warmer climates, or the warmer season in tropical climates. Bacterial pathogen removal is generally assessed by the removal of faecal coliforms (a pragmatic classification mostly, 90%, comprising *Escherichia coli*) and their excellent removal is attributed to a combination of high pH, high oxygen concentrations (from algal photosynthesis) and light which leads to rapid death by photo-oxidation (Curtis et al., 1992) and possibly high pH in its own right.

Unfortunately, the hyper-eutrophic conditions associated with death of typical bacterial pathogens and their indicators are classically associated with the growth of *Vibrio cholerae*. Thus it is at least plausible that disease causing toxigenic *V. cholerae* may grow in WSP (Anonymous, 199x). *V. cholerae* may be divided into three groups, the classical disease causing O1 serotype, the relatively novel O139 "Bengal" serotype and the other non toxigenic serotypes (collectively but incorrectly referred to as non O1 serotypes). The molecular basis of cholera toxicity is increasingly well understood. Nevertheless, it is apparent that the toxigenic and non toxigenic species are closely related. Thus, the alarming, but plausible hypothesis that WSP might be a source of, not a barrier to, cholera is supported by a number of studies of the fate of non-O1 *V. cholerae*. Non-toxigenic *V. cholerae* are relatively easily cultured in WSP and a number of authors have reported that such organisms grow vigorously in WSP, their numbers increasing in inverse proportion to the number of faecal coliforms (Lesne et al., 1992).

There have been earlier studies of the fate of toxigenic *V. cholerae*. However, these studies were unconvincing. They were essentially opportunistic in nature and consisted of relatively short studies of pilot scale systems that were receiving wastewater containing very low levels of the target organism. Moreover, the performance of WSP was assessed using a single culture base methodology of suspect, but undefined efficiency, and sensitivity in treatment systems. Since both lives and economies are at risk we have sought to assess the hypothesis that WSP are not barriers to *V. cholerae* by using the best available technology compatible with field conditions in a full scale system in an endemic region. Both culture and non-culture based methods were employed and the efficiency and sensitivity of the methodologies was established in an earlier study. All the approaches showed that the toxigenic and non-toxigenic organisms have different dynamics in WSP and that this form of WSP is indeed an efficient barrier for disease causing *V. cholerae* O1 and O139.

Methodology

Pagla wastewater treatment plant Serving about 18% of the City of Dacca this wastewater treatment plant consists of inlet works fed by a number of Archimedes screws running in parallel, a primary sedimentation tank and four baffled pond series running in parallel. During the study only two series were running. The flow was reported to be 163,000 m^3/day in the wet season and 122,000 m^3/day in the dry season.

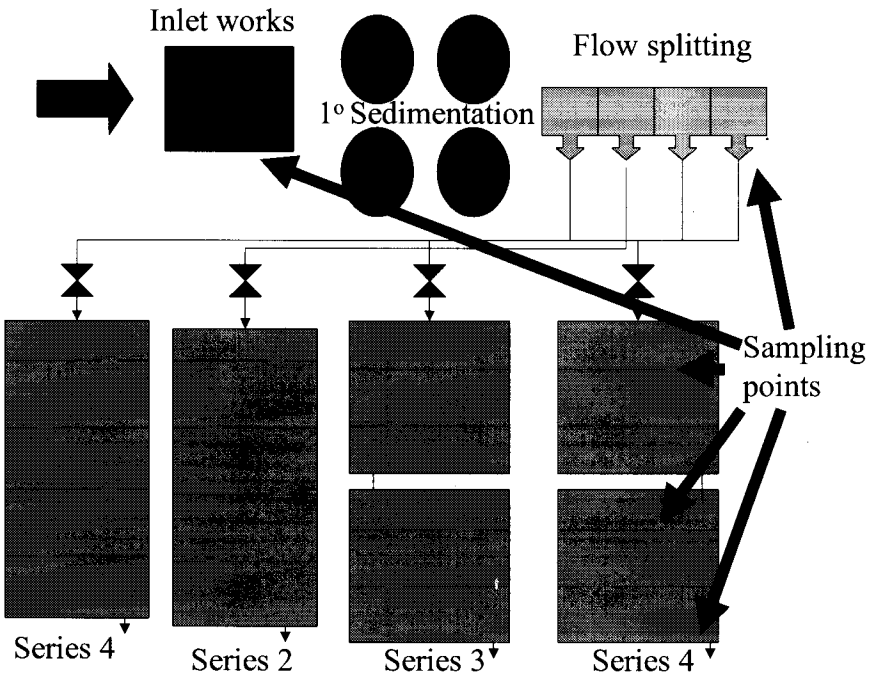


Figure 1. Sampling points at Pagla wastewater treatment plant. Only series 1 and 2 were receiving wastewater.

Sampling Samples were taken in the following order: final effluent; mid series; first pond; secondary sewage; raw sewage at approximately 11.00 each sampling day for 61 weeks commencing on the 7 of May 2000. Full microbiological analyses were undertaken at each sampling point. The microbiological methods are described in the section describing the methods development. Briefly, the non toxigenic *V. cholerae* were assessed by culture using a standard MPN format, the toxigenic organisms were quantified using: 5 tube MPN screened by examining both subcultures on TTGA plates (MPN culture) or PCR (MPN PCR) or FITC with monoclonal antibodies for O1 or O139.

Raw and settled sewage. An appropriate volume (2-3 litres) was taken and the total flow (inferred from the hours of operation of the pumps feeding the plant), BOD, pH (both as described in Standard Methods), mean air temperature of the current and previous week, mean number of hours of bright sunshine in the current and previous week, the mean rainfall for the current and previous week.

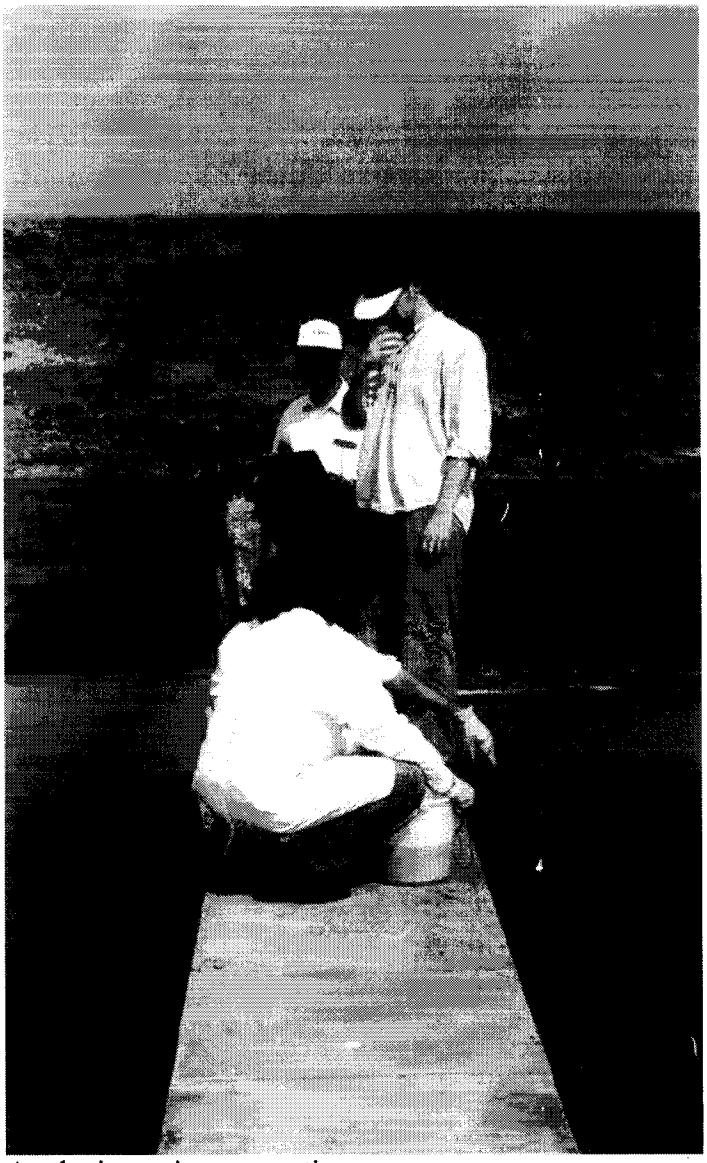


Figure 2. Sampling the first pond.

Pond Samples. At each sampling point DO, water temperature and pH at a depth of 10 cms, sunlight and salinity were measured. In addition a column sampler was employed and a sufficient number of column samples were collected to collect 20 litres of sample and return to the laboratory. The green algal concentration and the blue-green algal concentration and zooplankton (arthropod and rotifer) concentrations were determined by microscopic count.

The samples were refrigerated and transported promptly to the laboratory in the central Dacca.

Statistical treatment of the data. The statistical methods employed are described in a standard text (Sokal and Rohlf, 1996) and were undertaken using Minitab (Minitab, PA, USA). Special Matlab routines for Path Analysis are in preparation.

Results

Summary of performance. A 1 was added to the microbiological data before subjecting the results for each microbiological group to a Box-Cox Transformation. A natural log was selected as the consensus transformation.

The numbers of non-O1 and FC are consistent with earlier reports and comparisions of the fate of these organisms. Essentially no physical removal is observed in the conventional secondary sedimentation tanks followed by a consistent and statistically significant reduction ($p < 0.001$) over the pond series. By contrast, the non-toxigenic *V. cholerae* are not only not removed, but actually increase slightly through the WSP series.

The toxigenic *V. cholerae* are similarly unaffected by primary sedimentation. However, both culture based counts and direct counts of *V. cholerae* not only decrease but (figure 3.2) but appear to decrease more quickly than the faecal coliforms.

There are differences between the numbers and dynamics of the direct and culturable counts In particular the culture counts appear to plateau at a mean concentration of about 1 organism/100 ml. However, we have previously established that, with culture based counts in natural waters containing low numbers of organisms, there was no observable relationship between the numbers of organisms seeded and the numbers of organisms recovered.

The discrepancy between the direct counts and the culturable is typically of the order of 3 orders of magnitude. However, we had earlier established that even in fresh culture there is a discrepancy of an order of magnitude between the direct and culturable counts and that the recovery, by culture, of freshly seeded organisms rarely exceeded 1%.

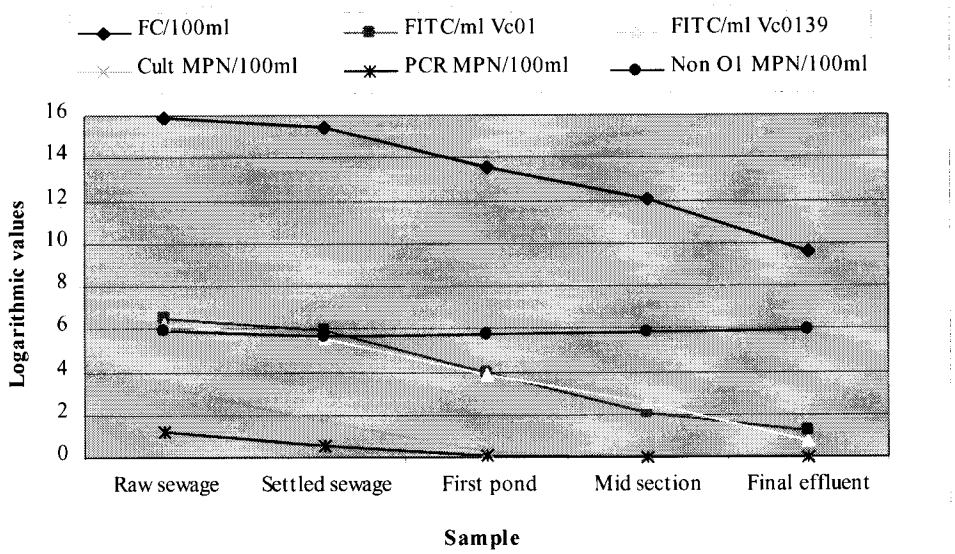


Figure 3.1. Logarithmic means of the pathogens and organisms at the different sampling points.

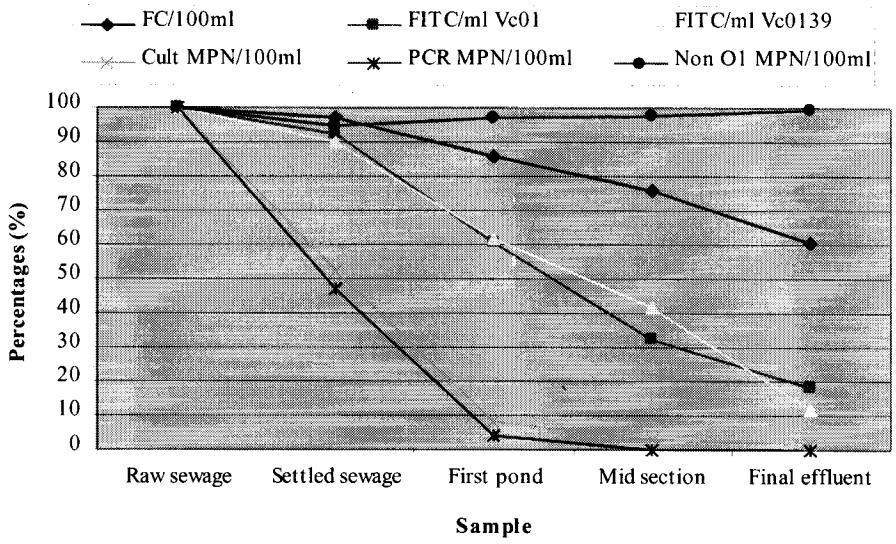


Figure 3.2. Logarithmic means of the pathogens and organisms at the different sampling points.

Table 3.1. Mean numbers of the different organisms in the different samples, with standard deviations in brackets and ranges in italics.

Organism	Raw sewage	Settled sewage	First pond	Middle section	Final effluent
Faecal coliforms	8.04×10^6 (5.64) *1.21×10^5- 1.97×10^8*	4.88×10^6 (8.00) *1.20×10^4- 5.94×10^7*	8.06×10^5 (10.1) *1.20×10^3- 2.67×10^7*	1.63×10^5 (11.9) *1.40×10^2- 6.58×10^6*	1.49×10^4 (14.3) *59.7 – 2.68×10^6*
FITC Vc01	6.45×10^2 (21.1) *0.00 - 2.69×10^4*	3.92×10^2 (23.8) *0.00 - 4.01×10^4*	51.4 (33.1) *0.00 – 2.16×10^4*	8.08 (24.0) *0.00 - 1.14×10^4*	3.29 (11.5) *0.00 – 3.13×10^3*
FITC Vc0139	4.93×10^2 (25.5) *0.00 - 4.44×10^4*	2.65×10^2 (19.5) *0.00 - 4.01×10^4*	45.2 (33.8) *0.00 – 2.97×10^4*	13.2 (23.8) *0.00 – 6.25×10^3*	2.06 (7.61) *0.00 - 1.88×10^3*
Culture	26.6 (11.0) *0.00 -1.60×10^3*	5.58 (9.58) *0.00 - 7.65×10^2*	1.30 (2.77) *0.00 – 1.41×10^2*	1.10 (1.70) *0.00 – 20.1*	1.05 (1.47) *0.00 – 20.1*
PCR	3.56 (4.53) *0.00 - 56.8*	1.81 (3.19) *0.00 - 62.2*	1.05 (1.48) *0.00 – 20.9*	0.00 (0.00) *0.00 – 0.00*	0.00 (0.00) *0.00 – 0.00*
Non01	3.80×10^2 (5.31) *0.00 - 1.60×10^3*	2.76×10^2 (6.62) *0.00 - 1.60×10^3*	3.17×10^2 (7.03) *0.00 - 1.60×10^3*	3.27×10^2 (6.89) *0.00 - 1.60×10^3*	3.65×10^2 (6.17) *0.00 - 1.60×10^3*

Correlelations Between Pathogens and Indicators The relationship between the different groups of bacteria were examined in two ways.

Firstly, through seeking a correlation between the individual counts for the organisms (table 1.2). Significant ($P < 0.001$) relationships were found between the all the pathogens and indicators with the exception of the non toxigenic *V.cholerae.* The correlations for the non-toxigenic *V. cholerae* are invariably low, and not statistically significant. The correlations between the other organisms are not high (<0.6) which implies that although the organisms are all declining the day to day dynamics are not tightly coupled.

This picture is accentuated when only mean logarithmic values are considered. In this case, we can see (table 1.3) that the non-toxigenic *V. cholerae* are entirely uncorrelated with either their pathogenic counter parts or the indicators, By contrast,

the average faecal coliforms counts are closely correlated with the average FITC counts of *V. cholerae* O1 and O139. The relationship between the culture based counts and faecal coliforms is less strong and it can be seen (figure 4) that this is attributable to the sporadic low counts in the former. It can also be seen that the decline in the serotype O139 is matched by the decline in O1.

Table 3.2. Table of the correlations between all the logarithmic values of the indicator organisms and of the pathogens (probability values in brackets).

	Faecal coliforms	FITC Vc01	FITC Vc0139	Culture	PCR
FITC Vc01	0.365 (0.000)				
FITC Vc0139	0.510 (0.000)	0.515 (0.000)			
Culture	0.398 (0.000)	0.517 (0.000)	0.453 (0.000)		
PCR	0.259 (0.000)	0.354 (0.000)	0.356 (0.000)	0.663 (0.000)	
Non01	0.095 (0.100)	0.133 (0.020)	0.055 (0.339)	0.121 (0.035)	-0.026 (0.654)

Table 3.3. Tables of the correlations between the means of the logarithmic values of the indicator organisms and of the pathogens (probability values in brackets).

MEANS	Faecal coliforms	FITC Vc01	FITC Vc0139	Culture	PCR
FITC Vc01	0.979 (0.004)				
FITC Vc0139	0.997 (0.000)	0.986 (0.002)			
Culture	0.819 (0.090)	0.881 (0.048)	0.857 (0.063)		
PCR	0.796 (0.107)	0.861 (0.061)	0.837 (0.077)	0.999 (0.000)	
Non01	-0.294 (0.631)	-0.227 (0.713)	-0.249 (0.686)	0.186 (0.765)	0.231 (0.708)

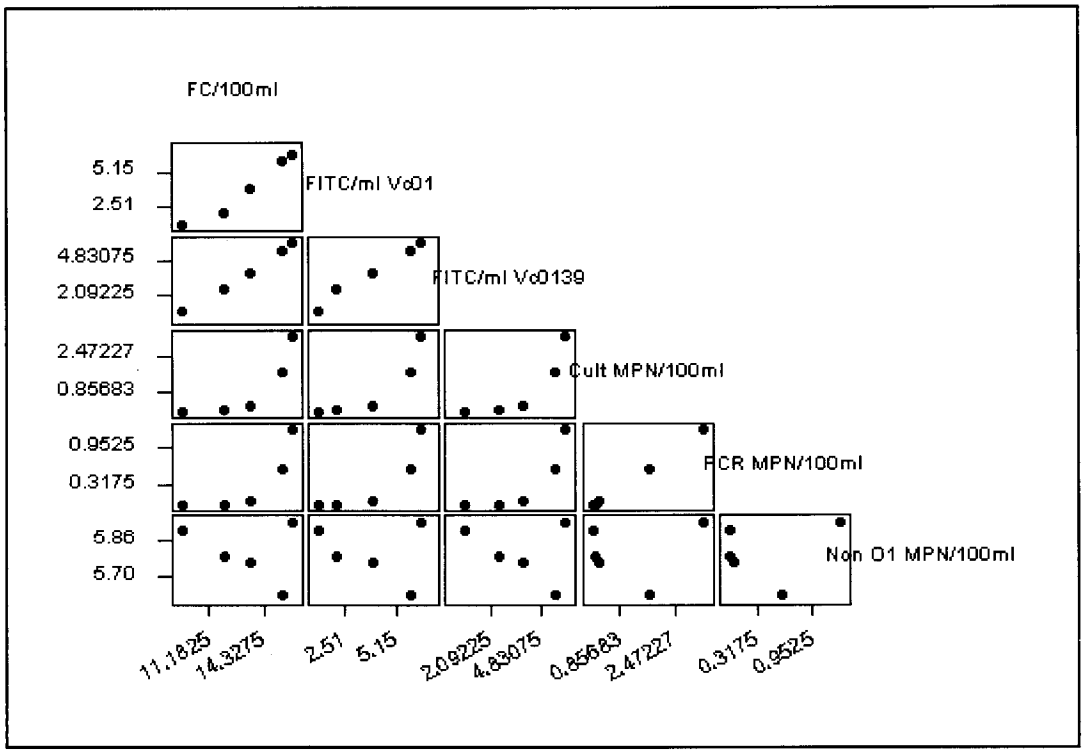


Figure 3. Regression graph of the relationships between the indicator organisms and the pathogens.

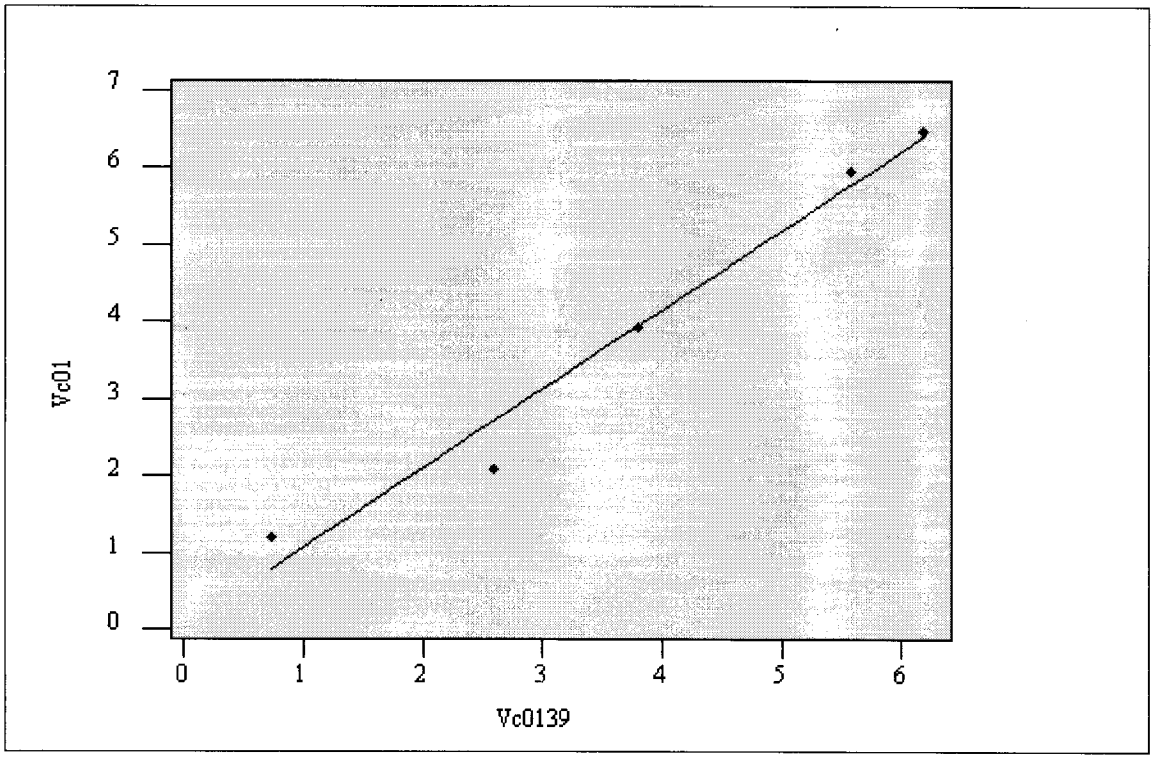


Figure 4. Regression plot of *V. cholerae* 01 versus *V. cholerae* 0139.

Table 3.4. Mean values and standard deviations of the parameters analysed in the samples of raw sewage and settled sewage.

Parameter		Raw sewage	Settled sewage
BOD_5 (mg/l)		9.58×10^2 ($\pm4.24\times10^2$)	7.28×10^2 ($\pm3.16\times10^2$)
pH		6.92 (±0.274)	7.06 (±0.253)
FC/100ml		2.14×10^7 ($\pm3.06\times10^7$)	1.42×10^7 ($\pm1.40\times10^7$)
FITC count/ml	**Vc01**	4.11×10^3 ($\pm5.67\times10^3$)	3.77×10^3 ($\pm7.32\times10^3$)
	Vc0139	4.19×10^3 ($\pm6.83\times10^3$)	2.40×10^3 ($\pm5.83\times10^3$)
Culture MPN/100ml		1.75×10^2 ($\pm3.53\times10^2$)	57.8 ($\pm1.35\times10^2$)
PCR MPN/100ml		9.98 (±15.1)	4.64 (±12.7)
Non01 MPN/100ml		8.05×10^2 ($\pm6.82\times10^2$)	7.12×10^2 ($\pm6.61\times10^2$)

Correlations Between Pathogens and Indicators and biological and physicochemical factors

The complete analysis of this data set is still underway. The data has been examined and normalised and correlated. Although, the data analysis is at a preliminary stage it is apparent that, although many sensible and plausible relationships have been observed, there are no obvious correlations between the pathogens and any biological or physical variable (tables 3.5-3.10).

Preliminary Discussion

The analysis of this data is still at a preliminary stage.

Nevertheless, we can unequivocally conclude that WSP can remove toxigenic *V. cholerae* O1 and O139. Moreover, the mean decline in indicator organisms is reflected in the mean decline in the O1 and O139 serotypes. However, the dynamics of the indicator organisms are probably not reflected in the dynamics of the pathogens.

Moreover, this decline was observed even though the non toxigenic representatives of the species not only persisted in the WSP. This suggests that the toxigenic and non-toxigenic *V. cholerae* do indeed have very different ecologies. The underlying reason for this difference between toxigenic and non-toxigenic strains is not at present clear. Moreover, we are far from confident that it will become apparent once all the statistical analyses are complete.

Notwithstanding the above, we are now confident that we can unequivocally confirm that WSP we have studied are barriers, indeed very effective barriers to the spread of *V. cholerae* O1 and O139.

Table 3.5. Arithmetic Mean values and standard deviations of the parameters analysed in the samples of the first pond, middle section and final effluent.

Parameter		First pond	Middle section	Final effluent
DO (mg/l)		1.31 (±1.17)	2.45 (±1.96)	4.04 (±1.77)
Water temp. (^{0}C)		28.8 (±3.53)	28.7 (±3.70)	28.4 (±3.80)
pH		7.41 (±0.310)	7.61 (±0.287)	7.80 (±0.240)
Sunlight (cm)		19.2 (±4.17)	20.4 (±5.26)	26.7 (±7.00)
Salinity (ppt)		0.444($\pm 7.19\times10^{-2}$)	0.449 ($\pm 6.49\times10^{-2}$)	0.420 ($\pm 7.03\times10^{-2}$)
FC/100ml		3.68×10^{6} ($\pm 5.64\times10^{6}$)	7.89×10^{5} ($\pm 1.23\times10^{6}$)	1.77×10^{5} ($\pm 4.66\times10^{5}$)
FITC count/ml	**Vc01**	1.76×10^{3} ($\pm 3.57\times10^{3}$)	1.59×10^{3} ($\pm 2.72\times10^{3}$)	5.98×10^{2} ($\pm 8.03\times10^{2}$)
	Vc0139	2.44×10^{3} ($\pm 6.07\times10^{3}$)	8.63×10^{2} ($\pm 1.28\times10^{3}$)	6.72×10^{2} ($\pm 5.60\times10^{2}$)
Zooplankton count/l	**Arthropod**	3.26 (±7.93)	67.6 ($\pm 2.03\times10^{2}$)	9.52×10^{2} ($\pm 1.94\times10^{3}$)
	Rotifer	3.39×10^{3} ($\pm 1.55\times10^{4}$)	4.53×10^{3} ($\pm 1.35\times10^{4}$)	4.34×10^{3} ($\pm 5.14\times10^{3}$)
	Total	4.20×10^{3} ($\pm 1.62\times10^{4}$)	5.19×10^{3} ($\pm 1.55\times10^{4}$)	5.91×10^{3} ($\pm 5.81\times10^{3}$)
Algal count/L	**Blue-green**	1.18×10^{5} ($\pm 2.26\times10^{5}$)	2.80×10^{5} ($\pm 4.63\times10^{5}$)	1.11×10^{6} ($\pm 1.30\times10^{6}$)
	Green	3.59×10^{6} ($\pm 7.95\times10^{6}$)	5.13×10^{6} ($\pm 9.13\times10^{6}$)	1.39×10^{6} ($\pm 1.71\times10^{6}$)
	Total	3.98×10^{6} ($\pm 8.09\times10^{6}$)	6.01×10^{6} ($\pm 9.53\times10^{6}$)	3.50×10^{6} ($\pm 2.41\times10^{6}$)
Culture MPN/100ml		4.64 (±21.4)	0.623 (±3.42)	0.328 (±2.56)
PCR MPN/100ml		0.344 (±2.69)	0.000 (±.0.000)	0.00 (±0.00)
Non01 MPN/100ml		7.80×10^{2} ($\pm 6.92\times10^{2}$)	8.33×10^{2} ($\pm 7.22\times10^{2}$)	8.87×10^{2} ($\pm 7.38\times10^{2}$)

Table 3.6. Table of the significant correlations observed in the first pond between the different parameters.

Significant correlation	Correlation value (p value)
DO and pH	0.393 (p=0.002)
DO and salinity	-0.385 (p=0.002)
DO and zooplankton	0.423 (p=0.001)
DO and arthropods	0.332 (p=0.009)
DO and rotifers	0.367 (p=0.004)
Feacal coliforms and temperature	-0.363 (p=0.004)
Feacal coliforms and salinity	0.419 (p=0.001)
Feacal coliforms and total algae	-0.456 (p=0.000)
Feacal coliforms and blue green algae	-0.479 (p=0.000)
Feacal coliforms and green algae	-0.464 (p=0.000)
Rotifers and pH	0.340 (p=0.007)
Rotifers and Zooplankton	0.857 (p=0.000)
Rotifers and Arthropods	0.402 (p=0.001)
Rotifers and total algae	0.509 (p=0.000)
Rotifers and blue green algae	0.387 (p=0.002)
Rotifers and green algae	0.525 (p=0.000)
Arthropods and zooplankton	0.333 (p=0.009)
Arthropods and total algae	0.408 (p=0.001)
Arthropods and blue green algae	0.421 (p=0.001)
Arthropods and green algae	0.395 (p=0.002)
Zooplankton and total algae	0.416 (p=0.001)
Zooplankton and green algae	0.413 (p=0.001)
Total algae and temperature	0.487 (p=0.000)
Total algae and pH	0.436 (p=0.000)
Total algae and blue green algae	0.632 (p=0.000)
Total algae and green algae	0.964 (p=0.000)
Blue green algae and temperature	0.512 (p=0.000)
Blue green algae and pH	0.411 (p=0.001)
Blue green algae and	0.683 (p=0.000)

green algae	
Green algae and temperature	0.555 (p=0.000)
Green algae and pH	0.469 (p=0.000)
VcO1 and VcO139	0.333 (p=0.009)

Table 3.7. Table of the significant correlations observed in the middle sectioin between the different parameters.

Significant correlation	Correlation value (p value)
Feacal coliforms and temperature	-0.418 (p=0.001)
Feacal coliforms and arthropods	-0.402 (p=0.001)
Feacal coliforms and total algae	-0.367 (p=0.004)
Feacal coliforms and blue green algae	-0.502 (p=0.000)
Feacal coliforms and green algae	-0.348 (p=0.006)
Zooplankton and DO	0.449 (p=0.000)
Zooplankton and temperature	0.446 (p=0.000)
Zooplankton and pH	0.364 (p=0.004)
Zooplankton and rotifers	0.960 (p=0.000)
Zooplankton and arthropods	0.542 (p=0.000)
Zooplankton and total algae	0.531 (p=0.000)
Zooplankton and blue green algae	0.408 (p=0.001)
Zooplankton and green algae	0.518 (p=0.000)
Rotifers and DO	0.388 (p=0.002)
Rotifers and temperature	0.437 (p=0.000)
Rotifers and pH	0.346 (p=0.006)
Rotifers and arthropods	0.492 (p=0.000)
Rotifers and algae	0.502 (p=0.000)
Rotifers and blue green algae	0.333 (p=0.009)
Rotifers and green algae	0.508 (p=0.000)
Arthropods and temperature	0.517 (p=0.000)
Arthropods and salinity	-0.361 (p=0.004)
Arthropods and total algae	0.466 (p=0.000)
Arthropods and blue green algae	0.560 (p=0.000)

Arthropods and green algae	0.421 (p=0.001)
Total algae and temperature	0.645 (p=0.000)
Total algae and VcO1	-0.336 (p=0.008)
Total algae and blue green algae	0.669 (p=0.000)
Total algae and green algae	0.969 (p=0.000)
Blue green algae and DO	0.418 (p=0.001)
Blue green algae and temperature	0.565 (p=0.000)
Blue green algae and green algae	0.600 (p=0.000)
Green algae and temperature	0.600 (p=0.000)

Table 3.8. Table of the significant correlations observed in the final effluent between the different parameters.

Significant correlation	Correlation value (p value)
DO and pH	0.374 (p=0.003)
Salinity and temperature	-0.493 (p=0.000)
Salinity and sunlight	-0.337 (p=0.008)
Salinity and faecal coliforms	0.342 (p=0.007)
Salinity and arthropods	-0.483 (p=0.000)
Salinity and blue green algae	-0.571 (p=0.000)
Feacal coliforms and temperature	-0.493 (p=0.000)
Feacal coliforms and arthropods	-0.479 (p=0.000)
Feacal coliforms and blue green algae	-0.572 (p=0.000)
Zooplankton and rotifers	0.871 (p=0.000)
Zooplankton and arthropods	0.492 (p=0.000)
Arthropods and temperature	0.738 (p=0.000)
Arthropods and blue green algae	0.687 (p=0.000)
Arthropods and green algae	-0.419 (p=0.001)
Total algae and green algae	0.750 (p=0.000)
Blue green algae and temperature	0.708 (p=0.000)
VcO1 and VcO139	0.390 (p=0.002)

Table 3.9. Table of the significant correlations observed between Feacal coliforms, VcO1, VcO139 and the sunlight×Secchi depth for the first pond, the middle section and the final effluent.

Sampling point	Significant correlation	Correlation value (p value)
First pond	Feacal coliforms with sunlight×Secchi depth	0.498 (p=0.000)
Middle section	Feacal coliforms with sunlight×Secchi depth	0.436 (p=0.000)

Table 0. Table of the significant correlations observed with faecal coliforms, VcO1 and VcO139 between samples of one sampling point in week x and the next sampling point in week x+1.

Sampling points	Significant correlation	Correlation value (p value)
Raw sewage with settled sewage	Feacal coliforms	0.640 (p=0.000)
Settled sewage with first pond	Feacal coliforms	0.669 (p=0.000)
First pond with middle section	Feacal coliforms	0.679 (p=0.000)
Middle section with final effluent	Feacal coliforms	0.479 (p=0.000)

Part 4: Risk Assessment

A provisional and conservative assessment of the risk of cholera transmission from a waste stabilisation pond in an endemic area

Introduction

Public health officials have the difficult task of determining when a wastewater is sufficiently clean to permit the irrigation of crops with a particular pathogen. Traditionally, safety has been equated with the complete absence of measurable pathogens. The apparent absence of the pathogen has been equated with the apparent absence of the hazard and thus the apparent absence of the risk. However, this approach may be flawed in one of two ways. Pathogens that are present but unmeasured may permit the transmission of disease from a "safe" effluent. Alternatively, safe water may be condemned because of the presence of pathogens at a low concentration or in a non-infectious physiological state (eg dead). The latter alternative is increasingly a consideration, as microbiological methods increase in sophistication and sensitivity. The need to distinguish between an actual risk and the potential risk was highlighted over 10 years ago when the WHO guidelines were first promulgated (Mara and Cairncross, 1989). The term potential risk is approximately synonymous with hazard in risk assessment terminology and is meant to refer to situations where a pathogen does not, or has not been proven to, cause disease. However, the only truly satisfactory manner in which to determine an actual risk is to conduct suitably planned, executed and analysed epidemiological studies in which pathogen levels are measured and disease outcomes assessed. Though such studies undoubtedly constitute the gold standard in risk assessment, they are difficult and expensive to carry out and can be constrained or, in certain circumstances, made untenable, by ethical considerations.

One alternative has been the use of simple risk assessment models which use experimentally derived dose response curves to infer the risk of infection from ingesting one or more organisms. Proposed in the 1980s these models do not employ the concept of the infectious dose. Instead they assume that the risk of infection is associated with the ingestion of a single organism.

The factors affecting the dose of the organism (volume ingested, survival in the environment, infectivity and so on) are all subject to some degree of variability. Thus an obvious way to improve the acceptability of such an approach is to randomly vary the components of the risk assessment model and so express the resulting risk as a distribution. A suitably conservative percentile can then be chosen to determine the level of risk.

Nevertheless, these models have never been corroborated for any pathogen in any scenario and are probably very conservative. Nevertheless, we have used this approach to assess the possible burden of infection that might hypothetically be imposed by an effluent from the Pagla WSP, if the WSP had been extended to meet the WHO standards.

Methods

The concentration of toxigenic *V. cholerae* in a Pagla pond meeting the WHO guidelines was estimated using the simple linear relationships between faecal coliform counts and the disease causing organisms.

The risk was estimated using the method of Haas modified by Sleigh to permit the random variation of: infectivity, dose, die-off in the environment, proportion infected and dose response relationship.

The exposure scenarios are essentially hypothetical and represent, at best, worst case guestimates. However, it known that in an infected community or family there are approximately 50 asymptomatic infections for each reported case of cholera and that, even in fresh pure culture, less than 10% of the organisms detectable under a microscope will multiply in culture.

Results and Discussion

We undertook a quantitative risk assessment in order to assess the risk of contracting cholera from wastewater irrigated with cholera containing wastewater.

The estimate of the risk is obviously sensitive to the numbers of pathogens assumed to be in the wastewater. Where culture based methods are used this value is obviously lower than if direct counts are employed. We used the work in the WSP to estimate that there were approximately 10^{-6} toxigenic *V. cholerae* detectable by MPN culture for each faecal coliform. On this basis we calculated that drinking 10-100 ml of treated wastewater a day carried an annual risk of about 1% of infection and a 0.03% risk of disease. Because this is a realativley low risk for a relatively extreme scenario we only looked in more detail at the risk estimates based on the more conservative direct FITCcounts.

The WSP did not achieve the WHO Guideline values. Nevertheless, there was an excellent linear relationship between the direct counts of the toxigenic *V. cholerae* (using FITC) and the faecal coliform counts (by culture).

For *V. cholerae* O1, the equation is:

Mean ln *V. cholerae* O1= -7.77298 + 0.87979 mean ln FC/100ml

Both the intercept and the coefficient were highly significant ($P< 0.001$) and the R^2 value was high (96%)

For *V. cholerae* O139, the equation is:

Mean ln FITC/ml Vc01 = -7.68991 + 0.862315 mean ln FC/100ml

Both the intercept and the coefficient were highly significant ($P< 0.001$) and the R^2 value was also high (99%).

The equations are reassuringly similar and predict geometric mean cholera concentrations (assessed using FITC direct counts) of 0.17/ml and 0.18/ml for serotype O1 and O139 respectively for an effluent achieving a geometric mean effluent concentration of 1000 faecal coliforms/ 100ml (the WHO guideline value). Nevertheless, direct counts are relatively conservative tools for the assessment of the numbers of bacteria capable of growth and therefore infection. In the laboratory, even in pure overnight cultures less than 10% of the organisms seen under the microscope would grow in the laboratory.

We have use these estimated concentration to estimate the risk of disease in various hypothetical scenarios (tables 1-4) assuming that either all or 10% of the organisms detected are capable of infection (tables 1-4, suffix a and suffix b) and assuming no prior immunity. This is somewhat conservative as the high levels of pathogen in the wastewater would only occur in an endemic region, but in an endemic region there would be significant prior immunity in the population. To add a further conservative element to the calculations we have used the upper 95 percentile of the probability distribution of the risk.

It is therefore perhaps unsurprising that we calculate that if all the organisms are infectious and one drinks a 100 ml of effluent a day then, over a year, there is a 100% probability of contracting a cholera infection (table 1a). However, if one accepts that not all the organisms are infectious, that exposure is less frequent and one uses disease rather than infection as the endpoint (table 1 b), then the probability of infection becomes quite low ($\sim 10^{-3}$ % at the 95 percentile).

We observed swimming and fishing in the lagoons at Pagla and note that where lagoons are used for aquaculture there may be close contact between workers and pond water. Again, if large doses are consumed (which seems unlikely) there is a good chance of contracting an infection. However, even quite frequent exposure to modest quantities of water carries a relatively low risk of disease (table 2a). Especially if some of the organisms detected are not infectious (table 2b).

The transmission of cholera by wastewater classically occurs because of the transmission of the disease by contaminated food crops. The model employed predicts that, if all the organisms observed were infective, daily exposure to 10 ml of effluent from a freshly irrigated food would carry an appreciable risk of infection and a 1% annual risk of disease (table 3a). However, this risk would drop appreciably if we assume that not all the organisms studied are infective and that some decay takes place prior to exposure. The risk of disease then drops to a relatively low level, especially if the frequency of consumption drops (table 3b).

Conclusion

The models used in this risk assessment are uncorroborated and, at best, give guidance of the nature of the likely risk and those factors that are most likely to be important. Conservative and simplistic risk assessment approaches of this kind are most helpful when they clearly show a risk to be low even in relatively extreme exposure scenarios. Thus the risk estimates based on culturable counts are relatively reassuring.

However, the calculations based on direct microscopic counts are less clear cut and suggest that issues such as post treatment die-off, the viability of the organisms detected and the volume and frequency of ingestion could materially affect the risk. This does not indicate that there is a significant risk of cholera transmission from wastewater meeting the WHO guidelines. However, it does suggest that further information would be helpful in the assessment of the further information would be helpful.

It would be particularly helpful to get some indication of the infectivity, or at least, viability of the organisms detected in latter part of the treatment process. However, this is problematic, not only because such organisms were rarely observed only 13 of the 61 samples were positive but also because this would probably require animal studies (the ligated loop test) or sophisticated histo-chemical studies.

Alternatively, it might be possible to quantitatively determine the risk by examining the relationship between direct counts of toxigenic strains of *V. cholerae* in water used for bathing in rural Bangladesh and disease. A study of this kind has been undertaken and the results are reported to be in preparation (Sack, personal communication). We anticipate that this data will allow us to substantially improve our risk assessment. In the mean time it is worthwhile noting mentioning an early attempt to relate direct counts of *V. cholerae* O1 to disease in which water sources for cholera cases were compared with water sources for controls (Huq, 1996). The concentrations of the pathogen were not reported, instead the proportion of positive samples was used. 50% of the control water supplies were positive for pathogenic *V. cholerae* by FITC, this compares favourably with the 20% of positives seen in the effluent of the Pagla treatment plant (even though the effluent quality was well above WHO guideline values).

Table 1a, Effluent used as drinking water all organisms infectious

Volume ingested	Fraction of Year Exposed	Reduction Prior to exposure	Proportion of infections resulting in disease	Upper 95% of annual risk of infection in % (standard deviation)
100	Daily	0	1	*100 ($\pm 4.11\times10^{-2}$)*
			0.02	*2.00 ($\pm 8.84\times10^{-4}$)*
		1	1	*64.1 (± 9.25)*
			0.02	*1.29 (± 0.187)*
	Weekly	0	1	*74.8 (± 8.02)*
			0.02	*1.51 (± 0.159)*
		1	1	*14.0 (± 3.37)*
			0.02	*0.283 ($\pm 6.89\times10^{-2}$)*
	Monthly	0	1	*28.5 (± 5.73)*
			0.02	*0.567 (± 0.113)*
		1	1	*3.46 (± 0.940)*
			0.02	*7.09×10^{-2} ($\pm 1.88\times10^{-2}$)*

Table 2b, Effluent used as drinking water, all organisms infectious

Volume ingested	Fraction of Year Exposed	Reduction Prior to exposure	Proportion of infections resulting in disease	Upper 95% annual risk of infection in % (standard deviation)
100	Daily	0	1	*64.2 (± 9.35)*
			0.02	*1.29 (± 0.186)*
		1	1	*10.1 (± 2.59)*
			0.02	*0.204 ($\pm 5.16\times10^{-2}$)*
	Weekly	0	1	*14.1 (± 3.46)*
			0.02	*0.282 ($\pm 7.11\times10^{-2}$)*
		1	1	*1.53 (± 0.407)*
			0.02	*3.09×10^{-2} ($\pm 8.61\times10^{-3}$)*
	Monthly	0	1	*3.51 (± 0.899)*
			0.02	*6.97×10^{-2} ($\pm 1.83\times10^{-2}$)*
		1	1	*0.355 ($\pm 9.42\times10^{-2}$)*
			0.02	*7.08×10^{-3} ($\pm 1.90\times10^{-3}$)*

Table 2a Contact through swimming or fishing all organisms are infectious

Volume ingested	Fraction of Year Exposed	Reduction Prior to exposure	Proportion of infections resulting in disease	Upper 95% of annual risk of infection in % (standard deviation)
1	Daily	0	1	*10.1 (±2.45)*
			0.02	*0.205 (±5.21×10^{-2})*
	Weekly	0	1	*1.54 (±0.407)*
			0.02	*3.03×10^{-2} (±8.28×10^{-3})*
	Monthly	0	1	*0.359 (±9.31×10^{-2})*
			0.02	*7.21×10^{-3} (±1.89×10^{-3})*
10	Daily	0	1	*64.5 (±9.15)*
			0.02	*1.28 (±0.183)*
	Weekly	0	1	*13.9 (±3.42)*
			0.02	*0.281 (±6.74×10^{-2})*
	Monthly	0	1	*3.48 (±0.924)*
			0.02	*7.07×10^{-2} (±1.82×10^{-2})*
100	Daily	0	1	*100 (±4.11×10^{-2})*
			0.02	*2.00 (±8.84×10^{-4})*
	Weekly	0	1	*74.8 (±8.02)*
			0.02	*1.51 (±0.159)*
	Monthly	0	1	*28.5 (±5.73)*
			0.02	*0.567 (±0.113)*

Table 2b Contact through swimming or fishing, 10% of organisms infectious

Volume ingested	Fraction of Year Exposed	Reduction Prior to exposure	Proportion of infections resulting in disease	Upper 95% annual risk of infection in % (standard deviation)
1	Daily	0	1	*1.08 (±0.279)*
			0.02	*2.12×10^{-2} (±5.67×10^{-3})*
	Weekly	0	1	*0.155 (±4.22×10^{-2})*
			0.02	*3.11×10^{-3} (±8.76×10^{-4})*
	Monthly	0	1	*3.58×10^{-2} (±9.15×10^{-3})*
			0.02	*7.07×10^{-4} (±1.88×10^{-4})*
10	Daily	0	1	*10.1 (±2.55)*
			0.02	*0.205 (±5.17×10^{-2})*
	Weekly	0	1	*1.53 (±0.412)*
			0.02	*3.03×10^{-2} (±7.99×10^{-3})*
	Monthly	0	1	*0.363 (±9.82×10^{-2})*
			0.02	*7.18×10^{-3} (±1.95×10^{-3})*
100	Daily	0	1	*64.2 (±9.35)*
			0.02	*1.29 (±0.186)*
	Weekly	0	1	*14.1 (±3.46)*
			0.02	*0.282 (±7.11×10^{-2})*
	Monthly	0	1	*3.51 (±0.899)*
			0.02	*6.97×10^{-2} (±1.83×10^{-2})*

Table 3a Irrigation with vegetables eaten raw, all organisms infectious

Volume ingested	Fraction of Year Exposed	Reduction Prior to exposure	Proportion of infections resulting in disease	Upper 95% annual risk of infection in % (standard deviation)
10	Daily	0	1	*64.5 (±9.15)*
			0.02	*1.28 (±0.183)*
		1	1	*10.2 (±2.54)*
			0.02	*0.205 ($\pm 5.18\times10^{-2}$)*
	Weekly	0	1	*13.9 (±3.42)*
			0.02	*0.281 ($\pm 6.74\times10^{-2}$)*
		1	1	*1.52 (±0.404)*
			0.02	*3.06×10^{-2} ($\pm 8.05\times10^{-3}$)*
	Monthly	0	1	*3.48 (±0.924)*
			0.02	*7.07×10^{-2} ($\pm 1.82\times10^{-2}$)*
		1	1	*0.354 ($\pm 9.46\times10^{-2}$)*
			0.02	*7.18×10^{-3} ($\pm 2.02\times10^{-3}$)*
100	Daily	0	1	*100 ($\pm 4.11\times10^{-2}$)*
			0.02	*2.00 ($\pm 8.84\times10^{-4}$)*
		1	1	*64.1 (±9.25)*
			0.02	*1.29 (±0.187)*
	Weekly	0	1	*74.8 (±8.02)*
			0.02	*1.51 (±0.159)*
		1	1	*14.0 (±3.37)*
			0.02	*0.283 ($\pm 6.89\times10^{-2}$)*
	Monthly	0	1	*28.5 (±5.73)*
			0.02	*0.567 (±0.113)*
		1	1	*3.46 (±0.940)*
			0.02	*7.09×10^{-2} ($\pm 1.88\times10^{-2}$)*

Table 3b Irrigation of vegetables eaten raw, 10% of organisms infective.

Volume ingested	Fraction of Year Exposed	Reduction Prior to exposure	Proportion of infections resulting in disease	Upper 95% of annual risk of infection in % (standard deviation)
10	Daily	0	1	*10.1 (±2.55)*
			0.02	*0.205 (±5.17×10^{-2})*
		1	1	*1.07 (±0.288)*
			0.02	*2.12×10^{-2} (±5.71×10^{-3})*
	Weekly	0	1	*1.53 (±0.412)*
			0.02	*3.03×10^{-2} (±7.99×10^{-3})*
		1	1	*0.156 (±4.24×10^{-2})*
			0.02	*3.06×10^{-3} (±8.52×10^{-4})*
	Monthly	0	1	*0.363 (±9.82×10^{-2})*
			0.02	*7.18×10^{-3} (±1.95×10^{-3})*
		1	1	*3.58×10^{-2} (±9.83×10^{-3})*
			0.02	*7.27×10^{-4} (±1.98×10^{-4})*
100	Daily	0	1	*64.2 (±9.35)*
			0.02	*1.29 (±0.186)*
		1	1	*10.1 (±2.59)*
			0.02	*0.204 (±5.16×10^{-2})*
	Weekly	0	1	*14.1 (±3.46)*
			0.02	*0.282 (±7.11×10^{-2})*
		1	1	*1.53 (±0.407)*
			0.02	*3.09×10^{-2} (±8.61×10^{-3})*
	Monthly	0	1	*3.51 (±0.899)*
			0.02	*6.97×10^{-2} (±1.83×10^{-2})*
		1	1	*0.355 (±9.42×10^{-2})*
			0.02	*7.08×10^{-3} (±1.90×10^{-3})*

Table 4a Playing Golf, all organisms infectious

Volume ingested	Fraction of Year Exposed	Reduction Prior to exposure	Proportion of infections resulting in disease	Upper 95% annual risk of infection in % (standard deviation)
1	Daily	1	1	*1.07 (±0.286)*
			0.02	*2.16×10^{-2} (±5.84×10^{-3})*
	Weekly	1	1	*0.152 (±4.04×10^{-2})*
			0.02	*3.09×10^{-3} (±8.24×10^{-4})*
	Monthly	1	1	*3.63×10^{-2} (±1.01×10^{-2})*
			0.02	*7.22×10^{-4} (±1.95×10^{-4})*

Table 4 b Playing golf, 10% of organisms infective

Volume ingested	Fraction of Year Exposed	Reduction Prior to exposure	Proportion of infections resulting in disease	Upper 95% of annual risk of infection in % (standard deviation)
1	Daily	1	1	$0.107 (\pm 2.87\times10^{-2})$
			0.02	$2.15\times10^{-3} (\pm 5.79\times10^{-4})$
	Weekly	1	1	$1.56\times10^{-2} (\pm 4.25\times10^{-3})$
			0.02	$3.07\times10^{-4} (\pm 8.34\times10^{-5})$
	Monthly	1	1	$3.58\times10^{-3} (\pm 9.81\times10^{-4})$
			0.02	$7.13\times10^{-5} (\pm 1.92\times10^{-5})$

Part 5: Dissemination

We believe that we have the information required to allow public health officials to confidently endorse the use of WSP in wastewater reuse for agriculture and aquaculture. What is now required is that the public health officials are aware of, understand, and most importantly, believe that the data we have acquired and our interpretation.

Long term credibility is ultimately derived from peer reviewed publication in prominent journals. In the short term this means we cannot publish the data informally on the web as many leading journals regard this as prior publication, which would disqualify us from prominent publication.

Nevertheless we have corresponded with practioners and researchers in South America, South East Asia and France (working in North Africa). We have been able to forward our preliminary results to these groups and have directed requests for a hands on training course in methodologies to ICDDR,B.

The papers will be published in the order that we have presented them in this paper.

The first (methods) paper will be presented to the IWA Conference on Health Related Microbiology in September (in South Africa). This conference attracts delegates from developed and developing countries and is expected to be particularly well attended by people from Africa generally. The paper will, subject to peer review, be published in *Water Science and Technology*.

The second (cholera concentration wastewater paper) is in an advanced state of preparation requiring only details of the incidence of cholera in Dacca at the time of sampling. This paper may be submitted for publication as soon as the methods paper has been submitted (to permit reference to the methodological issues). Ideally further information relating to the curious monthly period should be obtained and we are discussing this matter with colleagues at other institutions. However, we do not intend letting this matter delay submission.

The third (fate of *V. cholerae* in WSP) paper requires some further statistical analysis, though this is in hand and should be complete and submitted by the end of the year. We expect this manuscript to be well received as it shows the removal of putative "viable but non culturable" organisms from wastewater treatment system.

The fourth (risk assessment paper) is to be held back until the publication of complementary epidemiological data on the relationship between cholera and direct counts of the causative organism by Prof Sack's group. We believe that this paper will contain valuable information and Prof. Sack has told use he expects to submit this work shortly.

Finally, we have found the story of cholera in Bangladesh and Newcastle in the 1850s to be an invaluable tool for communicating with school children about the benefits of water and sanitation and other contemporary development issues. We have presented this work to school children of a wide variety of ages and backgrounds in meetings and presentations in a local museum, technical college and in Newcastle University.

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