VIROBATHE

METHODS FOR THE CONCENTRATION AND DETECTION OF ADENOVIRUSES AND NOROVIRUSES IN EUROPEAN BATHING WATERS WITH REFERENCE TO THE REVISION OF THE BATHING WATER DIRECTIVE 76/160/EEC

Project Number 513648

Specific Targeted Research Project

SSP-3

PUBLISHABLE FINAL

ACTIVITY REPORT

Period Covered: from 1.1.2005 to 31.3.2007
Start date: 1.1.2005 Duration: 27 months
Project Co-ordinator: Professor David Kay
Organisation: University of Wales Aberystwyth (UWA)

CONTENTS

INTRODUCTION

The Project VIROBATHE and its links to health questions still open at the entry into force of the Bathing Water Directive (BWD) 2006/07/EC

The Bacteriological Standards prescribed in BWD 2006/07/EC are health-related. This means that they are equivalent to a certain risk to contract gastroenteritis after bathing, this risk having been considered acceptable taking into account the health and recreational benefits of bathing.

The level of the bacteriological standards was estimated after conducting epidemiological studies. In these studies the probability of contracting gastroenteritis after bathing was related to the concentration of bacterial indicators present in the bathing water. As bacterial indicators are not pathogenic it was assumed that the concentration of bacterial indicators at which the gastroenteritis rate began to increase reflected a critical concentration of (probably viral) pathogens simultaneously present in the water. However, it is highly unlikely that the ratio of indicator bacteria to pathogens was identical in each and every one of the bathing sites under study. This ratio was rather an average of all bathing sites participating in the epidemiological studies, which implied that sometimes a certain concentration of indicator bacteria complying with the Directive might actually correspond to a concentration of pathogens considered not acceptable. For those cases it was felt that an indicator was needed which was more representative for pathogenic viruses than *E. coli* or intestinal enterococci.

The need of such a parameter is reflected by Article 14 of the BWD in which is stated that *the Commission shall, by 2008 submit a report… with particular regard to… scientific, analytical and epidemiological developments relevant to the parameters for bathing water quality, including in relation to viruses.* Virobathe has been directed at providing evidence to the Commission for this 2008 Report.

AIMS AND OBJECTIVES OF THE PROJECT

VIROBATHE has provided a technique for analysis of EU recreational waters for noroviruses and adenoviruses following comparison of methods for processing water samples to achieve the best virus recovery and detection consistent with cost and feasibility of use in routine monitoring laboratories. The target viruses (adenoviruses and noroviruses) were chosen since they both live in the human intestine, they are both shed in faeces. Adenoviruses are shed by healthy individuals, though some types can cause diseases. They are environmentally very resistant and these characteristics make them good candidates for a viral indicator. Noroviruses are the causative agent of most cases of adult viral gastroenteritis (and of many cases in children too). It is thus important to know as much as possible about the incidence of these pathogens in recreational waters.

Aims

1. To compare methods for norovirus and adenovirus detection in recreational waters.
2. To derive a combined concentration and detection technique to provide a reproducible system of testing recreational waters for the target viruses.

3. To provide evidence-based support for norovirus and adenovirus testing of environmental samples in respect of their role as the appropriate viral indicator of faecal pollution.
4. To prepare the technology for new Member States as part of the development of their environmental and social programmes.
5. To share technology between laboratories to achieve wider competence in the virological analysis of environmental materials.

The Project was done in two Phases; Phase 1 was the comparative evaluation of detection and concentration techniques to derive a method suitable for routine environmental monitoring and Phase 2 was the surveillance of recreational water sites using the combined method.

CONTRACTORS

The VIROBATHE Team comprised Scientists and Technicians in 16 Partner institutions across nine Member States as shown in the Table below:

Participant number	Participant name	Participant short name	Country
1	University of Wales	UWA	United Kingdom
2	Università di Pisa	PIS*	Italy
3	Department of Environment Food and Rural Affairs	CSL*	United Kingdom
4	Rijksinstituut voor Volksgezondheid en Milieu	RIVM*	Netherlands
5	Tor Vergata University	ROME TV	Italy
6	Landesgesundheitsamt Baden-Württemberg	LGA	Germany
7	University Henri Poincaré – Nancy	UHP-LCPME	France
8	Environment Agency	ENV	United Kingdom
9	University of Barcelona	UB*	Spain
10	Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	LGL	Germany
11	Umweltbundesamt	UBA*	Germany
12	National Veterinary Research Institute – State Research Institute	NVRI-SRI	Poland
13	Istituto Superiore Sanità	ISS	Italy

14	Faculdade de Farmácia da Universidade do Porto	UP	Portugal
15	State General Laboratory	SGL	Cyprus
16	Health Protection Agency	HPA*	United Kingdom

The Project Team was divided into *Evaluation Laboratories* (*6), which performed the first Phase of the Project in comparing detection methods and/or concentration methods for the target viruses, and *Surveillance Laboratories*, which carried out the surveillance of selected recreational water sites in Phase 2. All the Evaluation Laboratories also act as Surveillance Laboratories, so there were 15 Laboratories in the Surveillance Phase.

CO-ORDINATOR

The Co-ordinating Institution for VIROBATHE was the University of Wales, Aberystwyth, UK. The Responsible Scientist at UWA was Professor David Kay (dvk@aber.ac.uk,). The Lead Scientist and Vice Co-ordinator in charge of day to day running of the Project was Dr Peter Wyn-Jones (pyw@aber.ac.uk). In addition, there is a website www.virobathe.org on which was reported progress and where Standard Operating Protocols (SOPs), data and other material can be found as they are released into the public domain. This website will be maintained until at least the end of 2008. The SOPs will also be published in the peer-reviewed literature.

OVERVIEW OF PROGRESS

Period 1 (= Phase 1) focussed on the development of a combined concentration - detection procedure for the two target viruses specified by the Commission (adenovirus and norovirus).

The procedure for analysing water samples for viruses is technically more complex than for bacteria, which is comparatively simple. The virus method comprises two stages, (a) the concentration the viruses in a large (10 litres) volume of water into a volume of 10mL or less, and (b) the detection of the viruses in the small volume.

Detection techniques were assessed first. The Commission specified that a rapid test was developed and this implied the use of the Polymerase Chain reaction technique, which detects the specific genetic material of the target viruses. A test was also developed which would detect if any adenoviruses found were infectious. Quantitative detection by quantitative (real-time) PCR was also developed for adenoviruses and, as an extra non-scheduled part of the work, a real-time PCR for norovirus was included.

Concentration techniques were evaluated on a statistically validated comparative basis. From the many techniques available, five were selected on the basis of proven use in the field, experience of the laboratories, capital and recurrent cost, ease of use and perceived repeatability. Initially, one partner (HPA) evaluated the five techniques using adenovirus spiked into fresh and artificial seawater samples. Detection of recovered virus was assessed and replicate experiments were performed so that statistical analysis of the data could be done. Three methods were identified to take forward to the next stage, where five partner laboratories focussed on three of the methods tested earlier. The methods were:

1. Concentration by adsorption of virus to negatively charged membranes and elution with skimmed milk solution;
2. Concentration by adsorption to negatively charged membranes and elution with beef extract solution;
3. Concentration by adsorption to glass wool and elution with beef extract solution.

The laboratories evaluated the three methods using both target viruses, infectivity assay (for adenovirus) and (RT)PCR assay (for both viruses) and testing in both fresh water and artificial seawater. A decision was taken to modify the work in order to improve the quality of the data obtained by changing from a 'presence/absence' (RT)PCR detection procedure to one based on quantitative estimates. Though this increased the time required the Scientific & Technical Management Board agreed that the possibility of acquiring better quality data warranted the investment in time.

Period 1 Results

In comparison of the concentration methods it was apparent that there were few differences between the methods tested when evaluated for recovery of virus, whether detected by infectivity assay or molecular means. However, on the basis of statistical analysis of the data for fresh water matrices a concentration process of adsorption by membrane filtration followed by elution with skimmed milk solution gave the best recovery. For salt water there was even less difference between the methods tested but that adsorption by membrane filtration followed by elution with beef extract solution was marginally better.

It was also apparent that the recovery of virus was variable between laboratories and between samples. Comparative evaluation of virus recovery methods had not been done this way before and it was interesting to see that replicate samples could yield variable virus recovery values. This is clearly an issue for attention in developing the robustness of the method in Surveillance Laboratories.

The other main output from this period has been the Standard Operating Protocols (SOPs). These are deliverables and will be placed on the public area of the website following Commission approval.

The work of Period 2 was to implement the methods successfully developed in Period 1 for the detection of enteropathogenic viruses in recreational waters through structured programmes of field surveillance, reporting, and data analysis. This provided evidence-based support for norovirus and adenovirus testing of environmental samples in respect of their role as viral indicators of pollution. Additionally, Period 2 work transferred the technology to new Member States by virtue of their membership of the Consortium, and to a wider audience through the Final Conference and structured dissemination activities.

Successful completion of Period 2 activities provided scientific evidence which will assist policymakers and regulators in determining the feasibility of taking the research forward with reference to Article 14 of the Bathing Water Directive 2006 and any subsequent revision of the Bathing Water Directive (Directive 2006/7/EC) where it may be considered appropriate to include a viral parameter.

The first Period 2 activity was to train all scientists and technical staff in the combined concentration/detection procedure. This was accomplished in a four-days

Training Workshop (WP6.1) held at RIVM in Bilthoven. Hands-on experience was provided by the expert staff at RIVM and the scientists who had developed the methods in Period 1. At the same time, presentations were given by all Participants on the recreational water sites they had selected for surveillance later in the year. Following the Workshop, the methods were practised by all Participants in their own laboratories so they achieved competence before the Surveillance Phase. This included the analysis of blinded QC samples and resolution of problems due to local conditions such as water types being inhibitory to the (RT-)PCR reactions.

The Surveillance Phase was done over five months and included the 2006 Bathing Season. Recreational waters were chosen to reflect (a) current recreational activity, including boating, swimming, surfing etc. and (b) potential pollution. Sites were not necessarily designated Bathing Water sites. Sampling was usually done weekly, four 10-litre samples being taken for virological analysis at the same time and from the same place on each occasion. A additional samples was taken for QC purposes and samples were also taken for bacterial faecal indicator organism (FIO) analysis. Samples were processed and analysed by the standard techniques. Results were reported to the WP Leader using a standard spreadsheet. The WP Leader sent the results to the Co-ordinator who reviewed all the data and assisted in the resolution of problems.

Viruses were found in many of the samples taken across the Consortium Laboratories and processed by the methods developed in the Evaluation Phase, and so the principal aim of the Project was thus achieved. There was no laboratory that did not find one or other of the target viruses in at least some of their samples. Virus occurrence ranged widely, from no noroviruses being found in some laboratories (e.g. SGL, in Cyprus) to some laboratories finding high frequencies of virus occurrence, such as the UHP laboratory in Nancy, France.

The recovery methods worked as expected and confirmed the confidence placed in the choice of techniques. In total 1544 samples were taken for virological analysis. Out of the 1544 samples, 1410 were processed by the standard methods derived during the Evaluation stage. The remainder were processed using the method modified for concentration of marine water samples, since the Project Team regarded on-going development as imperative if techniques were to be evaluated thoroughly and the best methods derived. The modification of the marine water concentration protocol was introduced during the surveillance and results from its use suggest that it may be useful in concentrating viruses from clean waters.

Overall, 39.2% of samples were positive for one or more viruses. A breakdown of the analysis of samples processed by SOPs 1 and 2 is shown in Table 6:

Table 1: Percentage of positive samples in recreational waters tested by SOPs 1 & 2

	Positive samples	Total samples	% positive
Fresh water	402	928	43.3
Marine water	151	482	31.3
Total samples	553	1410	39.2

A summary of the results is shown in Figure 1. As expected, adenoviruses were detected most often, with 36.4% of samples being positive for one or more adenoviruses. The test did not select for non-human adenoviruses, nor did it distinguish between types of human adenovirus, though the nucleic acid of about 10% of positive samples was sequenced in WP3.2 and showed a range of strains. It was not surprising to find over a third of the samples positive for adenoviruses since they are frequently shed by humans showing no symptoms of infection.

Many groups of workers have found large numbers of positive samples in several studies, and it is known that the virus is resistant to environmental breakdown. Several samples were positive for types 40 and 41, which are associated with gastrointestinal disease in children.

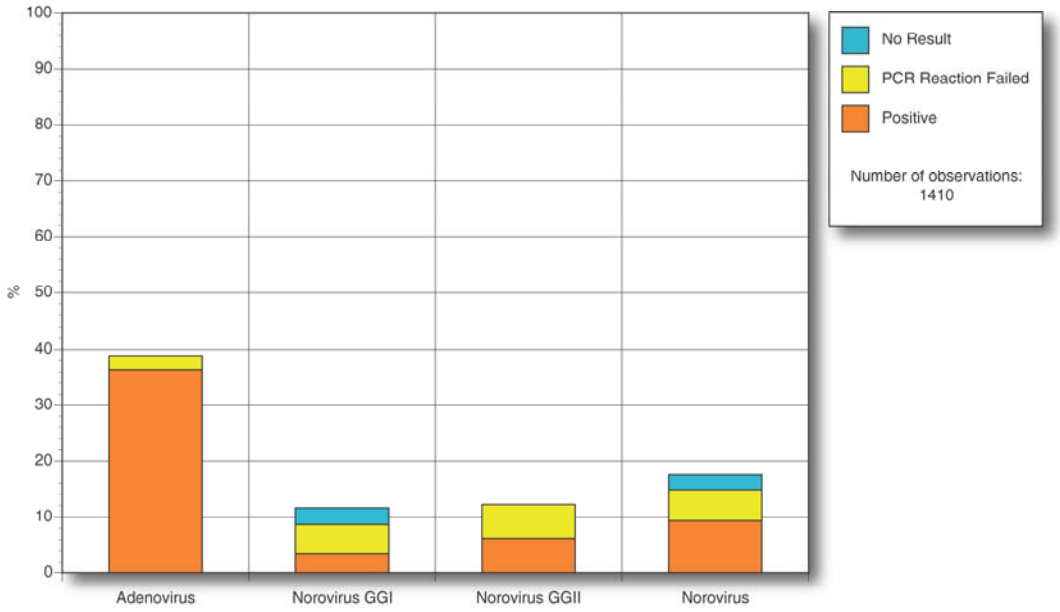

Figure 1: Summary of virus detection in all water types

About 9.4% of samples were positive for noroviruses, and these were divided between the two genogroups (GG) of norovirus. Norovirus GGI was found in 3.5% of samples and norovirus GGII in 6.3% (Figure 1). The presence of GGII noroviruses was expected, based on other smaller studies and experience. It is known that detection of GGI noroviruses in the environment is not matched by their detection in clinical samples, and this contributed to the view that many norovirus infections are symptomless, with GGI viruses being particularly under-represented among those found in clinical cases.

In addition to (RT-)PCR analysis for presence/absence of target viruses done in all Laboratories, a selection of samples was also analysed by specialist Laboratories (UB and ISS) by QPCR and sequence analysis to provide an estimate of the quantity of virus present in certain locations and to give a picture of the strains or serotypes in the samples.

All Laboratories were also required to analyse a selection of their samples by the infectivity assay for adenoviruses.

The data were analysed statistically to determine the extent and robustness of associations between the virus occurrence and faecal indicator data. This was necessary to provide (a) a picture of the pollution status of the surveillance sites in terms of the recognised bathing water Directive parameters and (b) to take forward the process of linking virus occurrence to a parameter demonstrated to be related to health effects, and thus to begin to determine if the target viruses can be used as a viral indicator.

Analysis by categorical and parametric statistics showed that there were significant associations between adenovirus occurrence and FIO levels in fresh waters and that it may be possible to link virus occurrence to enterococci levels and thus to a parameter recognised as a measure of health risk. Sample numbers were too low to be certain of such associations for marine waters.

The evidence from VIROBATHE now provides a springboard for further work to link virus occurrence with health effects of recreational water use, either through correlation with enterococci or by epidemiological studies based on projects such as Epibathe. VIROBATHE has shown that the idea of using viruses as an indicator of pollution and as a valid parameter in a Bathing Water Directive has moved from the "conceptual" at the beginning of the Project through the "possible" stage reached at the end of Phase 1, where the methods were shown to work in the laboratory, to the "feasible" stage, where structured field studies have furnished evidence that viruses can be detected in surveillance programmes. The next stage will be to move from "feasible" to "operational", where quantitative determinations of target viruses (probably adenoviruses) will be reconciled with levels of known faecal indicators (probably enterococci) so that meaningful discussions on the formulation of a viral parameter based on sound scientific data can be held.

OUTCOMES OF THE EXPERIMENTAL WORK

The Aims of the Project were more than fulfilled in respect of:

- Training of scientific and technical staff in the concentration and detection of the target viruses at a hands-on four days workshop
- Multicentre trialling of the methods in all Consortium Laboratories
- Establishment of Quality Assurance systems in Laboratories
- Sampling and analysis of recreational water samples over a five months period I the summer of 2006
- Analysis for target viruses, faecal indicator organisms, bacteriophage and physico-chemical parameters
- Reporting of data to the Project Co-ordinator for collation and statistical analysis
- Statistical analysis to determine any relationships between virus occurrence and faecal indicator organism presence ion the field samples
- Reporting on procedures, findings, and outcomes to the Final Conference and Technology Transfer Workshop, and to the Commission.

OUTCOMES IN RELATION TO LINKS TO HEALTH QUESTIONS

The principal aim of Virobathe was directly related to the requirements of Article 14 and therefore focused on developing a reliable reproducible method to detect both adenoviruses and noroviruses in bathing water. The final aim was to enable filling the monitoring gap recognized in the epidemiological studies. This principal aim was subdivided into the following tasks:

1. To find a method to concentrate adenoviruses and noroviruses from 10 liters of bathing water into a smaller volume, amenable to further analysis by the detection method.
2. To develop a PCR and RT-PCR procedure to be used with the concentrates obtained with task 1. The goal was a presence/absence method.
3. To evaluate the viability of the combined methods (concentration/PCR and concentration RT-PCR) for the monitoring of bathing waters and to establish a relationship between the fraction of virus-positive samples found in each of the sample sets collected and the concentration of bacterial indicators.
4. A subsidiary task was to explore if the quantitative Real Time PCR procedure (as opposed to the presence/absence procedure) for the detection of adenovirus could be modified and applied for the monitoring of bathing waters.

The following table gives the principal locations in the Report of the tasks mentioned above:

Task	Subdivision of the task	Main reference	Remarks
1	**Concentration method for fresh** waters based on adsorption of the viruses to cellulose nitrate and elution with beef extract	Period 1 Report pp24,28 (WP5.1&5.2)	discarded
1	**Concentration method for fresh** waters based on adsorption of the viruses to cellulose nitrate and elution with skimmed milk	Period 1 Report pp24,28	discarded
1	**Concentration method for fresh** waters based on adsorption of the viruses to glass wool and elution with skimmed milk	Period 1 Report pp24,28	discarded
1	**Concentration method for fresh** waters based on adsorption of the viruses to glass wool and elution with beef extract	Period 1 Report pp24,28	**Method of choice**
1	**Concentration method for coastal** waters based on adsorption of the viruses to cellulose nitrate and elution with beef extract	Period 1 Report pp24,28	discarded
1	**Concentration method for coastal** waters based on adsorption of the viruses to cellulose nitrate and elution with skimmed milk	Period 1 Report pp24,28	**Method of choice**
1	**Concentration method for coastal** waters based on adsorption of the viruses to glass wool and elution with skimmed milk	Period 1 Report pp24,28	discarded
1	**Concentration method for coastal** waters based on adsorption of the viruses to glass wool and elution with beef extract	Period 1 Report pp24,28	discarded

[continued...]

2	Development and tuning of a PCR protocol for the **detection of adenoviruses**	Period 1 Report p16 (WP2)	
2	Development and tuning of a RT-PCR protocol for the **detection of noroviruses**	Period 1 Report p17	
3	Evaluation of the feasibility of the combined concentration/detection method for the monitoring of bathing waters: **results of the participating laboratories for adenoviruses**	Period 2 Report p28	
3	Evaluation of the feasibility of the combined concentration/detection method for the monitoring of bathing waters: **results of the participating laboratories for noroviruses**	Period 2 Report p28	
3	Evaluation of the feasibility of the combined methods (concentration/PCR and concentration RT-PCR) for the monitoring of bathing waters: **Correlation between the concentration of *E.coli* and adenovirus**	Period 2 Report p28	
3	Evaluation of the feasibility of the combined concentration/detection method for the monitoring of bathing waters: **Correlation between the concentration of intestinal enterococci and adenovirus**	Period 2 Report p41	
3	Evaluation of the feasibility of the combined concentration/detection method for the monitoring of bathing waters: **Correlation between the concentration of *E.coli* and norovirus**	Period 2 Report p41	
3	Evaluation of the feasibility of the combined concentration/detection method for the monitoring of bathing waters: **Correlation between the concentration of intestinal enterococci and norovirus**	Period 2 Report p41	
4	Exploring the quantitative Real Time PCR procedure for the detection of adenovirus and its application for the monitoring of bathing waters	Period 2 Report p84	Substantial inhibition of the reaction observed

CONCLUSIONS IN RELATION TO LINKS TO HEALTH QUESTIONS

1. Virobathe has enabled developing a presence/absence method for adenoviruses and noroviruses in several liters of bathing water, which has been found viable *in praxi* .
2. The positive correlation between the concentration of adenoviruses and other viral (coliphages) and bacterial indicators warrant plausibility of the results.
3. A substantial number of samples shown standard indicator values complying with the Bathing Water Directive were positive for adenoviruses.
4. Point 3 is an analytical corroboration of the epidemiological finding according to which in some waters the rate of gastroenteritis among bathers begins to rise at indicator values well below the allowed standard values.

QUESTIONS REMAINING

In order to pinpoint the concentration of adenoviruses representing a hazard for bathers, further development of the Quantitative PCR (QPCR) for adenoviruses is strongly recommended.

As the presence of inhibitors in the extracts is the main reason for the shortcomings shown by the QPCR, further work is necessary to eliminate these inhibitors from the extracts. When this is achieved, a combined epidemiological/analytical study is indicated to find out the relationship between the concentration of adenoviruses and the rate of gastroenteritis.

GENERAL CONCLUSIONS OF THE PROJECT

1. It has been shown that it is feasible to introduce the Virobathe-developed virus concentration and detection methods into virology laboratories of a wide range of skills and expertise.
2. Target viruses could be concentrated by relatively inexpensive techniques, which should therefore be adaptable to routine environmental monitoring laboratories and to laboratories in countries with low resource provision.
3. The chosen concentration method was applicable across all the fresh waters – France to Poland.
4. Adenoviruses and noroviruses were present in fresh and marine recreational waters and were detectable with varying frequency.
5. Target viruses were widely present in environmental samples across Europe.
6. Adenoviruses were found to be statistically associated with human faecal pollution in fresh water.
7. The nPCR for adenovirus is reliable.
8. At least some of the samples contained infectious adenovirus as detected by the ICC-PCR method.
9. Detection, especially for adenoviruses, can be quantified by a quantitative PCR technique.
10. Norovirus were found not to be a practical target for surveillance as they were not sufficiently abundant nor was the RT-PCR robust.
11. More work is needed to optimise a method for marine waters.
12. The RT-PCR for norovirus GII worked well but a means of monitoring process and assay efficiency other than by the use of IACs should be investigated.
13. Further work on nucleic acid extraction is needed to optimise recovery.
14. Further work is needed on reducing inhibition of the PCR by components, especially in fresh water.
15. Human adenoviruses are common, the serotypes of virus vary, but adenovirus 41 is common. Norovirus GGII was detected more often than GGI, but the difference in detection rates was not as great as is seen in clinical settings.

16. The evidence from VIROBATHE now provides a springboard for further work to link virus occurrence with health effects of recreational water use, either through correlation with enterococci or by epidemiological studies based on projects such as Epibathe. VIROBATHE has shown that the idea of using viruses as an indicator of pollution and as a valid parameter in a Bathing Water Directive has moved from the "conceptual" at the beginning of the Project through the "possible" stage reached at the end of Phase 1, where the methods were shown to work in the laboratory, to the "feasible" stage, where structured field studies have furnished evidence that viruses can be detected in surveillance programmes. The next stage will be to move from "feasible" to "operational", where quantitative determinations of target viruses (probably adenoviruses) will be reconciled with levels of known faecal indicators (probably enterococci) so that meaningful discussions on the formulation of a viral parameter based on sound scientific data can be held.

Management activities proceeded in parallel with scientific ones. Core Management Team (CMT) and Scientific & Technical Management Board (STMB) meetings were held at appropriate intervals during Period 2. STMB contributed greatly to the successful outcomes of the Project and guided it throughout both Periods. CMT provided overall Project management and was also critical in providing the framework essential to final successful outcomes of the project. The Project Advisory Board (PAB) which had met in Bilthoven at the Period 1 to Period 2 interface, met again during the Final Conference and expressed itself well pleased with the progress, outcomes and management of Virobathe.

In general, Virobathe has clearly fulfilled the requirements of the Commission and the objectives set by the Team. Significant progress has been made towards the provision of a method for detecting waterborne enteropathogenic viruses in recreational waters across Europe, and this work should act as a springboard for later studies directed at formulating a viral standard for bathing waters in the future.

* * *