Surveillance of adenoviruses and noroviruses in European recreational waters

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ABSTRACT

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1. Introduction

Enteric viruses have been frequently implicated in recreational water-related gastro-intestinal (GI) disease (Sinclair et al., 2009). Studies in Europe and the US suggest that most infections contracted as a result of swimming, canoeing or other recreational use of sewage-polluted

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water may be viral in nature (e.g. Medema et al., 1995; Gray et al., 1997). Enteric viruses may cause asymptomatic or mild infections in humans, but these faecal-orally transmitted viruses may also cause more serious disease, such as hepatitis and meningitis, especially in vulnerable groups, e.g. young children (Nwachuku and Gerba 2006). Enteric viruses are recognized as agents that can cause large outbreaks throughout the world with thousands of cases (Sarguna et al., 2007; Bucardo et al., 2007; Iijima et al., 2008; Zhang et al., 2009). Novel emerging viruses such as SARS coronavirus, human parechovirus and zoonotic influenzaviruses also appear to be excreted in faeces but enteric transmission is not always clear (Ding et al., 2004). Transmission routes for enteric viruses may be diverse such as person-person, food- or waterborne associated with insufficient hygiene and sanitation (Fields Virology 5th ed. 2007). Disease outbreaks associated with enteric viruses, such as enteroviruses, noroviruses and astroviruses, in bathing water have been described (Hauri et al., 2005; Maunula et al., 2004). However, bathing water related outbreaks may be easily missed due to unidentified source and agent.

Enteric viruses present in water may originate from discharges of raw or treated sewage, run-off of animal manure or directly by humans or animals. Viruses commonly associated with waterborne disease include the human adenoviruses (HAdVs), noroviruses (NoVs), hepatitis A and E viruses (HAV, HEV), parvoviruses, enteroviruses, and rotaviruses (RV). In addition, sewage, especially from slaughterhouses, may contain (for example) animal adenoviruses, sapoviruses, and HEV, which may be zoonotic. Viruses originating from (un)treated sewage can contaminate bathing water after discharge onto surface waters (in)directly used for recreational water activities. All are capable of infection by ingestion. Some multiply in the intestine and may cause diarrhoea and /or vomiting, while some are associated with tissues, e.g. the liver, other than the gut. The viruses responsible for waterborne infections are not usually identified at the time of a disease outbreak following recreational water activity, and robust associations between the simultaneous presence of virus in faeces of affected individuals and in the water are only occasionally demonstrated. The epidemiological picture of disease associated with recreational use of water is therefore far from complete, and measures to limit enteric disease after exposure to recreational water are based on water quality parameters built on the detection of faecal bacterial indicator organisms (FIOs). However, it has been shown that water conforming to bacterial standards may contain high levels of human enteric viruses and that FIOs often fail to predict the risk for waterborne pathogens including enteric viruses (Gerba et al., 1979; Lipp et al., 2001).

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Further, several studies have shown that levels of indicator bacteria do not correlate with those of viruses, particularly when faecal indicator concentrations are low (Contreras-Coll et al., 2002). Viruses are known to be more resistant to environmental degradation than bacteria (Vasl et al., 1981; Thurston-Enriquez et al., 2003; Rzeżutka and Cook, 2004; de Roda
5 Husman et al., 2009). Together with the understanding that GI illness may be due to viruses rather than bacteria, this provides a case for using a viral indicator of human faecal pollution rather than to rely exclusively on bacterial parameters.

Bathing water quality in the European Union (EU) has been regulated since 1976 by the Bathing Water Directive (76/160/EEC). In 2006 (Anon, 2006) this was revised by including
10 enterococci (and, in fresh waters, *Escherichia coli*) as the principal microbial determinants which placed the microbiological parameters on a firmer scientific footing (Kay et al., 1994, 2004: Wiedenmann et al., 2006; WHO, 2003) and allowed classification of bathing waters to be undertaken with more confidence. When tested at sufficient frequency *E.coli* may indeed be a good indicator of faecal pollution and therefore of the probability of waterborne disease.
15 However, in the EU Directive the frequency is only about once in two weeks and testing takes two days.

The earlier Directive included an enterovirus parameter which stipulated that 95% of 10 l water samples taken during the bathing season should contain no (zero p.f.u.) enteroviruses. This was based on early work (Farrah and Bitton, 1990) which suggested that, for poliovirus,
20 Coxsackie A and Coxsackie B viruses, between one and twenty virus infectious units might be sufficient to cause infection. The pathogenesis of enterovirus infections is now better understood, and this belief is considered unsound in determining water quality. Further, the presence of enteroviruses does not necessarily correlate with the presence of important pathogens such as hepatitis A virus (Dubrou et al., 1991; Pina et al., 1998). The enterovirus
25 parameter was removed during the revision of the 1976 Directive.

Concentrations of some viruses in surface waters can be determined by cell culture monolayer plaque assays, but the technique is not applicable to most viruses of prime interest. Furthermore, cell culture is expensive and time-consuming, and detection of viruses is now done mainly by molecular methods such as (reverse transcription (RT))-PCR or nucleic acid
30 sequence-based amplification (NASBA) which amplify RNA / DNA. Although mainly described as end point assays, amplification products of both techniques can be detected by real-time methods. The major advantages of real-time detection are low detection limits and

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hands-on time, and the ability to quantify amplification products, which is very important in being able to estimate the public health risks of low levels of enteric viruses in bathing water.

A viral indicator may be better suited to indicate the risk of human pathogenic viruses in bathing waters. However, cell culture-based methods for viral detection were deemed to be too costly, requiring too much expertise and specialized equipment and with too long a turnaround time. For this reason, the EU Framework 6 Project VIROBATHE was done to devise a robust, rapid and cost-efficient method for routine compliance monitoring of enteric viruses in recreational waters. Part of the work involved Europe-wide surveillance of recreational waters to determine the frequency of target virus occurrence and, to a limited extent, serotypes and quantities. To provide context in which the virus levels may be judged, the work included determination of virus occurrence in recreational waters together with FIOs and phage levels to provide general water quality data. The viruses selected as targets were adenoviruses and noroviruses. The former are shed by many individuals (often without showing symptoms), they have been found in surveys of polluted waters (e.g. Pina et al., 1998; Laverick et al., 2004; Lee et al., 2004; Miagostovich et al., 2008), they are more environmentally robust than enteroviruses (Enriquez et al., 1995; Thurston-Enriquez et al., 2003) and, being DNA viruses, their detection by PCR does not have the problems associated with the genetic variation seen with RNA viruses. They are also more likely to be detected in recreational water samples (e.g. Pina et al., 1998; Miagostovich et al., 2008), especially if sensitive nucleic acid detection methods are used, and they may therefore provide the best indicator of viral faecal pollution. Noroviruses are the most important cause of acute viral gastroeneritis in people of all age groups and many waterborne oiutbreaks have been reported (e.g. Hoebe et al., 2004; Hewitt et al., 2007).

The study reported here was performed to demonstrate that a common concentration method could be used across recreational waters in widely diverse geographical areas, that viruses concentrated by this method could be detected by a rapid molecular method, that it was possible to enumerate viruses and to investigate whether there was a range of sero/genotypes of the target viruses present across the locations studied.

2. Materials and Methods

2.1 Survey Design

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Each of the 15 Surveillance Laboratories located in nine countries selected up to two sites for study which were sampled during the EU Bathing Season 2006, and samples were concentrated and analysed by molecular means for the target viruses. FIOs and various physico-chemical parameters were also determined. Data were sent to the co-ordinating Laboratory at the University of Aberystwyth for collation.

2.2 Sampling Sites

Each laboratory selected up to two sites (main site and second site) for the study (Table 1 and Figure 1) insert Table 1 and Figure 1. The principal criterion for a site being chosen was its current use for recreational water activity; sites were not chosen on the basis of being EU-designated bathing waters, nor because they had a history of pollution in the area, though several sites were known to be impacted by sewage effluent. A minimum of 80 ten-litre water samples from the main site was taken and up to 20 additional samples were taken in the event of (e.g.) heavy rain or when investigators considered that there was some other occurrence which may have resulted in deterioration of water quality. The second site could also be used if the main site yielded negative data in the first stages of sampling, or for taking the 20 additional samples following the 80 minimum to be taken at the main site. Thus, each laboratory could focus on one site (100 samples) or divide surveillance between the main site (80 samples) and the second site (20 samples). In practice both approaches were used, so in total, 24 sites were sampled. Sites were sampled at approximately weekly intervals from the end of May to the beginning of November 2006, which included the Bathing Season in all Member States. On each sampling occasion, four 10 l samples (a 'tetrad'), plus one additional sample for positive Quality Control (QC) purposes, were collected from each site. One 250 ml sample for somatic coliphage analysis and one 250 ml sample for bacterial faecal indicators were also taken. In total each laboratory processed and analysed at least 100 water samples for virus detection and 25 samples for bacterial and phage enumeration.

2.3 Sample Processing

Many methods for the concentration and detection of enteric viruses in water samples have been described (Wyn-Jones and Sellwood, 2001). For virological water quality to be assessed on a comparable basis, a single method common to all laboratories was needed for each water type (fresh or coastal/transitional) analysed during the surveillance programme.

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Prior to the surveillance stage several different methods were evaluated and the best in terms of virus recovery, ease of use and capital/recurrent costs was selected.

2.3.1 Concentration of fresh water samples by glass wool filtration

For freshwater samples a modification of the glass wool method (termed the Fresh Water Method, FWM) of Vilaginès et al., (1993) was used. The glass wool filter was made by compressing 10 g glass wool (type 725; Rantigny, Saint-Gobain, France) into a 30 cm by 3 cm polystyrene column to obtain a filter height of 6-8 cm. The filter was washed by gravity with 50 ml volumes of (in order) 1 M HCl, tap water, and 1 M NaOH, followed by tap water until the filtrate pH was neutral. Water samples (10 l) were conditioned with 1 M or 0.1M HCl to pH 3.5 to enhance binding of the viruses to the filter and passed through the filter at a rate not exceeding 1.5 l min^{-1}. When all the sample had passed through the filter the virus was eluted from the glass wool by slow (20-30 min) passage of 200 ml 3% (w/v) beef extract at pH 9.5 in 0.05 M glycine buffer through the filter. The eluate was flocculated by the addition of 1 M and 0.1 M HCl until the pH reached 3.5. The resultant protein floc, containing virus, was deposited by centrifugation at 7,500 x *g* for 30min, dissolved to a final volume of 10 ml phosphate buffered saline (PBS) and stored at -20°C pending further analysis.

2.3.2 Concentration of marine water samples by nitrocellulose membrane filtration

Coastal/transitional water samples were processed by filtration through nitrocellulose membranes, elution and organic flocculation (Marine Water Method, MWM; Wyn-Jones et al., 2000). The sample, at pH 3.5, was passed through a 142 mm diameter glass fibre pre-filter and a nitrocellulose membrane in a Sartorius filter holder at a maximum rate of 1.5 l min $^{-1}$. The filtrate was run to waste and the virus was then eluted from the membrane by slow passage (10min) of 200 ml skimmed milk solution (0.1% in 0.05M glycine buffer). The eluate was flocculated by reducing its pH to 4.5 with M HCl and centrifuging as above.

2.4 Extraction of nucleic acids from sample concentrates

Nucleic acid (NA) was extracted from 5 ml volumes of sample concentrate using the NucliSens® miniMAG™ system (Biomérieux, France) according to manufacturer's instructions, with slight modifications comprising centrifugation at 1500 x *g* for 2 min after addition of the silica suspension to reduce the chance of cross contamination. The final

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100 µl NA extract was centrifuged at 13,000 x *g* for 1 min to pellet any remaining traces of silica which could inhibit downstream (RT-)PCR reactions, the supernatant was transferred to a clean microfuge tube and was stored at -80°C if not used immediately.

2.5 Human adenovirus PCR

For the detection of human adenovirus in the water samples the nested PCR based on the method of Allard et al., (2001) was employed, using primers Hex1deg and Hex2deg for the first round of amplication and primers nehex3deg and nehex4deg for the second round. Additionally, an internal amplification control (IAC, see below) was incorporated in the assay, and a carryover contamination prevention system utilising uracil N-glycosylase (UNG) in the first round PCR and dUTP (replacing dTTP) in both PCRs. The reaction incorporated a hot-start polymerase (Platinum® Taq DNA polymerase, Life Technologies Inc.).

The target amplicon sizes were 301 bp in the first round and 171 bp in the second round. The first round reaction conditions were as follows: 10 µl NA, 1 X Platinum® Taq buffer, 1.5 mM Mg^{++}, 250 µM dNTPs, 0.5 µM primer Hex1deg, 0.5 µM primer Hex2deg, 1U Platinum® Taq (Life Technologies Inc.), and 1 U HK-UNG (Epicentre®, Madison, Wisconsin). Five µl IAC were added in the first round. Adenovirus DNA (20 ng μl^{-1}), and ultrapure water were included as positive and negative reaction control, respectively. After 10 min at 50°C (UNG) and 10 min at 95°C (Activation of Taq polymerase), cycling conditions included 45 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by a final extension of 72°C for 5 min. The second round reaction conditions were: 1 X Platinum® Taq buffer, 1.5 mM Mg^{++}, 100 µM dNTPs, 0.5 µM primer nehex3deg, 0.5 µM primer nehex4deg, and 1U Platinum® Taq. Two µL from the first round reaction were used as target. The thermocycling conditions were 94°C for 3 min, then 45 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by a final extension of 72°C for 5 min. The amplicons were electrophoresed in a 2% agarose gel stained with 10 ng ml^{-1} ethidium bromide or equivalent nucleic acid staining methods such as SYBR-Gold, and subsequently visualised by UV transillumination

2.6 Norovirus RT- PCR

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To detect norovirus, the nested RT-PCR based on the method of Vennema et al., (2002) was used, and comprised amplification of norovirus RNA-dependent RNA polymerase (RdRp) gene sequences by RT-PCR followed by a semi-nested PCR for each genogroup (G). Depending on the laboratory, contamination carryover prevention was also incorporated utilising uracil N-glycosylase (UNG) in the PCR. The target amplicon sizes were 327 bp in the RT-PCR, 188 bp in the G I nested PCR, and 237 bp in the G II nested PCR.

Reverse transcription PCR conditions were as follows: 1 X OneStep buffer (Qiagen, UK), 400 µM each dNTP, 1 X OneStep enzyme mix (Qiagen, UK), 0.5 µM primer JV12Y, 0.5 µM primer JV13i, and 50 U RNasin (RNasin®Plus, Promega, UK), 1U Platinum® Taq (Life Technologies Inc.). Five µl IAC were added in the first round. A 10 µl sample of nucleic acid was used as target. The thermocycling conditions were 50°C for 30 min, 95°C for 15 min, then 40 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 1 min, followed by a final extension of 72°C for 10 min. The second round PCR conditions were as follows: 1 X Platinum® Taq buffer, 2.0 mM Mg^{++}, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 0.4 µM primer JV12Y, 0.4 µM primer Ni-R, 1 U HK-UNG and 1U Platinum® Taq. One µl from the first round reaction was used as target. The thermocycling conditions were 50°C for 10 min, 95°C for 10 min, 96°C for 3 min then 40 cycles of 95°C for 1 min, 40°C for 1 min and 72°C for 1 min, followed by a final extension of 72°C for 10 min. The amplicons were electrophoresed in a 2 % agarose gel stained with 10 ng ml^{-1} ethidium bromide or equivalent nucleic acid staining methods such as SYBR-Gold, and subsequently visualised by UV transillumination.

2.7 Internal amplification controls (IACs)

The need to guard against false negative reactions required the use of a novel IAC in each PCR. For adenovirus IACs, oligonucleotides were constructed which contained the adenovirus primer sequences used in each round flanking primer sequences for amplification of *invA* sequences from *Salmonella enterica* (Malorny et al., 2003; Malorny et al., 2004). The amplicon was cloned into a plasmid (pGem T-Easy vector) by Yorkshire Bioscience Ltd (York, UK). The resulting pADENOIAC plasmid was linearised at the unique *Pst*I site downstream of the adenovirus IAC insert region. Yorkshire Bioscience supplied pADENOIAC in 100 µl volumes containing 1 mg ml^{-1} plasmid DNA in 10 mM Tris-HCl, 1

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mM EDTA buffer pH 8.0. The IAC amplicon sizes were 384 bp in the first round and 337 bp in the second round.

For the norovirus IAC the RNA was synthesized by the addition of complementary sequences of the first round primers JV12Y and JV13i to part of the β-globin gene, resulting in a PCR
5 product of 369 base pairs. In this same construct sequences complementary to the GGI nested-primer Ni-R and to the GGII nested-primer GI were included. The construct was subsequently cloned downstream of a T7 RNA-polymerase promoter. The RNA IAC was prepared by Yorkshire Bioscience Ltd (York, UK) using plasmid pnJV IAC which was linearised with Sa1I restriction endonucleases and purified. The resulting RNA was
10 transcribed using the T7 RNA polymerase transcription system. Template DNA was removed from the preparation during incubation with RNase-free DNase. The RNA was purified by LiCl precipitation followed by multiple phenol / chloroform extractions. The preparation was concentrated to 1.0 $\mu g\ \mu l^{-1}$ by precipitation with ethanol and dissolving in a minimal volume of MilliQ / 18.2 MΩ quality water.

15 Amplification products of the IAC with the G I specific primers produced a PCR product of 228 base pairs, G II-specific amplification resulted in a PCR product of 277 base pairs. The working concentration of each IAC (in 10 mM Tris-HCl, 1 mM EDTA buffer pH 8.0, plus 500 ng ml^{-1} bovine serum albumin) was empirically determined as the dilution which consistently (triplicate determinations) gave a positive signal. Aliquots of the adenovirus
20 IAC were stored at –20°C for and at –70°C for the norovirus IAC.

2.8 Infectivity detection

At least 10 adenovirus-positive (by nested-PCR) samples from each Laboratory were tested for virus infectivity by integrated cell culture-PCR (ICC-PCR, Reynolds et al., 2001; Greening, Hewitt and Lewis, 2002). If any of the four test samples in a tetrad was positive by
25 human adenovirus nested-PCR then the sample concentrate which had given the strongest PCR band was tested for infectious adenovirus by inoculation of cell cultures and observation over five days for the development of a cytopathic effect (c.p.e.) indicative of virus multiplication. No infectivity assay was performed if the adenovirus nested-PCR on all four concentrates was negative. At least two 25cm^2 flasks, each containing a monolayer of
30 confluent A549 cells (European Collection of Cell Culture, ECACC, UK) were inoculated with 1 ml of sample concentrate. At least one flask was incubated for five days (T=5). One

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flask was analysed without incubation (T=0), to guard against detection of seed virus. One negative control with cell culture medium only was set up. Following incubation, flasks in the first set (T=5) were frozen and thawed three times and the separated supernatant analysed by the adenovirus nested PCR.

5 *2.9 Faecal indicator organisms and somatic coliphage*

Somatic coliphages were detected using the double agar layer method as described in ISO 10705-2:2001. Samples were tested undiluted and diluted 1/10 according to ISO 10705-2:2001. Detection of *Escherichia coli* and intestinal enterococci was done according to ISO 9308-3 and ISO 7899-1 using Microtiter plates. One laboratory enumerated bacteria by
10 colony-forming units (c.f.u.).

2.10 QPCR assay for the detection of HAdV DNA.

Virus nucleic acid in at least 10 samples which were positive for adenovirus by nested-PCR from each Laboratory was quantified by real-time qPCR. Assays were done in 25-µl reaction mixtures each containing 10 µl of DNA and 15 µl of TaqMan® Universal PCR Master Mix
15 (Applied Biosystems) containing 0.9 µM of each primer (AdF and AdR) and 0.22 5µM of fluorogenic probe (AdP1) as previously described (Hernroth et al., 2002).

Following activation of the uracil-N-glycosylase (2 min, 50ºC) and activation of the AmpliTaq Gold for 10 min at 95ºC, 45 cycles (15 s at 95ºC and 1 min at 60ºC) were performed.

20 A pBR322 plasmid containing the HAdV 41 hexon sequence kindly donated by Dr. Annika Allard from the University of Umeå, Sweden, was used to construct a standard containing 10^1 to 10^7 copies of DNA in the 10 µL added to the PCR reaction. Each dilution of standard DNA suspensions was run in triplicate. Ten µL of undiluted and a ten-fold dilution of the DNA suspensions obtained from water samples were run in duplicate. In all QPCRs carried
25 out, the amount of DNA was defined as the mean of the data obtained. A non-template control (NTC) and a non-amplification control (NAC) were added to each run.

2.11 Sequence analysis

The amplicons obtained after nested-PCR assays of HAdV or NoV were purified using the QIAquick PCR purification kit (QIAGEN, Inc.). Purified DNA was directly sequenced with

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the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 with Ampli Taq® DNA polymerase FS (Applied Biosystems) following the manufacturer's instructions. The conditions for the 25-cycle sequencing amplification were: denaturing at 96ºC for 10 s, annealing for 5 s at 50ºC and extension at 60ºC for 4 min. The nested primers
5 were used for sequencing at 0.05 µM concentration.

The results were checked using the ABI PRISM 377 automated sequencer (Perkin-Elmer, Applied Biosystems). The sequences were compared with the GenBank and the EMBL (European Molecular Biology Library) using the basic BLAST program of the NCBI (The National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/).
10 Alignments of the sequences were carried out using the ClustalW program of the EBI (European Bioinformatics Institute of the EMBL, http://www.ebi.ac.uk/clustalw/).

2.12 Quality Assurance – robustness of the concentration and detection methods

The robustness of the methods was calculated using the results obtained from the analysis of quality control samples. Nine laboratories participated in the trial of the methods for analysis
15 of fresh waters and six laboratories participated in the trial of the methods for analysis of marine samples. Test samples comprised 1ml aliquots of adenovirus Type 2 and norovirus GII-4 which were added by the participants to their own water samples. A batch of adenovirus Type 2 and a batch of norovirus GII-4 was prepared, distributed into single-use ampoules and sent to each participant. On each sampling occasion 1 ml of the adenovirus
20 Type 2 and 1 ml of the norovirus positive control material was added to a separate 10 l quality control sample of the recreational water being tested. Negative samples were prepared from a mixture of de-ionised and tap water, or artificial seawater. Each participant analysed at least 25 sets of quality control samples.

The raw data sent by each laboratory were statistically analysed according to the
25 recommendations of Scotter et al., (2001) by the methods of Langton et al., (2002). The *diagnostic sensitivity* was defined as the percentage of positive samples giving a correct positive signal, and *diagnostic specificity* was defined as the percentage of negative samples giving a correct negative signal. *Accordance* (repeatability of qualitative data) was defined as the percentage chance of finding the same result, positive or negative, from two identical
30 samples analysed in the same laboratory under predefined repeatability conditions, and *concordance* (reproducibility of qualitative data) was defined as the percentage chance of

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finding the same result, positive or negative, from two identical samples analysed in different laboratories under predefined repeatability conditions. These calculations take into account different replication in different laboratories by weighting results appropriately. The *concordance odds ratio* (COR) was the degree of inter-laboratory variation in the results, and
5 expressed as the ratio between accordance and concordance percentages (Langton et al., 2002). The COR value may be interpreted as the likelihood of getting the same result from two identical samples, whether they are sent to the same laboratory or to two different laboratories. The closer the value is to 1.0, the higher is the likelihood of getting the same result. Confidence intervals for accordance, concordance and COR were calculated by the
10 method of Davidson and Hinckley (1997); each laboratory was considered representative of all laboratories in the "population" of laboratories, not just those participating in this analysis.

3. Results

The study surveillance period ran from the end of May until early November 2006. Nine participant Laboratories collected samples at both of their sampling sites, whereas six
15 Laboratories took samples from only their main site. Thirteen fresh water sites and 11 marine sites were sampled. A total of 1410 samples was taken of which 928 were from fresh water and 482 were from marine sites.

3.1. Virus detection

Overall, 582 out of 1410 samples (41.3%) were positive for one or more of the target viruses
20 (Figure 2) insert Fig 2. Adenoviruses were detected more often than noroviruses, 513 (36.4%) samples being positive for one or more human adenovirus types, while 132 samples (9.4%) tested positive for one or both norovirus genogroups; these were divided between G I 49 (3.5%) and G II 88 (6.2%, Figure 2). Five samples (two marine and three fresh water) were positive for both norovirus genogroups. Out of 513 human adenovirus-positive
25 samples, 63 (12.3%) were also positive for one or both NoV genogroups (33 out of 381 fresh water samples and 30 out of 132 marine samples). Just four samples (two fresh water and two marine), were positive for all three virus types.

3.2. Water type

Adenoviruses were detected more often in fresh water (381 adenovirus-positive samples out
30 of 928, 41.1%, Figure 3) than in marine water (132 out of 482, 27.4%) insert Fig 3.

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Conversely, noroviruses (either G I or G II or both) were detected less often in fresh water samples (58 norovirus-positive samples out of 928, 6.3%) than in marine water (79 out of 482, 16.4%, Figure 3). Further, in marine waters the detection rate of norovirus G I was almost as high as norovirus G II (7.9% compared with 8.5%), which differs from the clinical
5 context where G I viruses are found much less frequently than G II types in patients from gastroenteritis outbreaks, even in surveys of unaffected individuals (e.g. Verhoef et al., 2009). However, these high G I detection rates were mainly due to just four sites having higher frequencies of NoV G I.

3.3. Variation according to site

10 Virus occurrence ranged widely between sites (Table 2) insert Table 2. Some Laboratories reported no viruses at all in any sample while others found many samples positive for at least one virus. Human adenoviruses were detected in all except two sites, one marine and one fresh water. Sites were chosen on the basis of their recreational use, and most were impacted by sewage effluent. The range of sewage contamination impact was reflected in the different
15 frequencies with which the target viruses were detected (Table 3). insert Table 3 Among the marine sites, 55% of samples from Pomezia, Rome, were positive for HAdV, while none was found at Barcelona, and none was detected at Larnaca, Cyprus, where it is known no sewage is discharged. Among the freshwater sites, no HAdV was found at Kirchentellinsfurt Lake in Baden-Württemberg, while 80% of samples were HAdV-positive at Amper Grasslfing in
20 Bavaria and 91% were positive at the site at Tomblaine, Nancy, a site well known for its recreational activities (mainly canoeing) but also known to have recognised anthropogenic effects. With respect to noroviruses, five out of 11 marine water, and four out of 13 freshwater sites gave samples positive for G I noroviruses. The frequencies for G II noroviruses were eight from both types of site. Overall, the data showed that adenoviruses
25 were present at more sites than noroviruses.

Most sites had between 0 and 25% samples virus-positive in respect of both adenoviruses and noroviruses. To illustrate the distribution of sites relative to the frequency of virus detection, Figures 4 (marine sites) and 5 (fresh water sites) insert Figs 4 and 5 show the frequencies of positive samples divided into four groups (0-25% 26-50%, 51-75% and 76-100% samples
30 positive) plotted against the number of sites in each group. Thus there were, for example, six of the 11 marine sites which reported between zero and 25% samples being HAdV-positive, three sites between 26% - 50% and 3 sites between 51% - 75% (Figure 4). There were

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several sites where the adenovirus frequency fell into the higher categories and two fresh water sites where over 76% samples were HAdV positive.

Examination of the marine water norovirus G I data, when divided according to sites, shows that almost all the norovirus G I-positive samples (37/38) were found in four sites in Italy, the only other norovirus G I-positive marine water sample being found in Portugal. There was no evidence of outbreaks of norovirus-related disease in the areas local to the detection of G I virus in the environmental samples at the time when the samples were taken.

3.4. Virus infectivity ICC-PCR

From each Laboratory, at least 10 samples that gave a strong HAdV positive signal by nested PCR were analysed further by inoculation into cell culture and analysis of the supernatants by PCR. Fifty-one of 482 marine sample concentrates and 226 of 928 freshwater sample concentrates were tested. The results are shown in Table 4.insert Table 4. Twenty-four (47%) of the marine water samples were found to be positive by nested PCR following inoculation of A549 cell cultures and where uninoculated control cultures remained negative, and where cultures inoculated and sampled immediately after inoculation also remained negative. Forty-six (20%) fresh water samples were positive for infectious HAdV.

3.5. Relationship of virus frequency to faecal indicators and phage DK/MW

This section is being revised by David Kay and Mark Wyer

3.6. QPCR assay for the detection of HAdV DNA.

A total of 132 marine and freshwater samples which had previously tested positive by nested-PCR were further analysed by the QPCR assay of Hernroth et al., (2002). Eighty (60.6%) samples were positive, with a mean value of 3260 genome copies (GC)/ l of water. The percentage of positive samples was similar in both types of recreational water; 61.3% positive for fresh water with mean GC values of 558 GC/l versus 58.6% positive for marine waters with mean concentrations of 8810 GC/l.

3.7. Analysis of the sequence of the PCR products obtained.

Fifty-three samples were further analysed to type the HAdV present. HAdV serotypes 12, 31, 40 and 41 were the more frequently detected in 4, 8, 4 and 22 samples respectively.

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Other serotypes observed with lower frequency were 19 and 1. Serotypes 1, 2, 3, 12, and 31 were observed after analysing 7 samples which had been cultured in A549 cells as part of the infectivity detections.

Nineteen samples were studied for determining NoV genotypes. Fifteen were confirmed as G II, with seven of them being G II.4. Four were G I, with one being G I.2. Over the last few years the most newly emerging NoV strains belong to GII.4 and show a global presence (Bull et al., 2006; Rowena et al., 2006).

3.8. Robustness of the methods

The results of the robustness calculations of the adenovirus / freshwater method are shown in Table 5 for the adenovirus / seawater method in Table 6, the norovirus / freshwater method in Table 7, and the norovirus / seawater method in Table 8. Insert Tables 5-8. With the adenovirus / freshwater method the diagnostic sensitivity, or percentage of correctly identified positive samples, was 77.2 %, and the concordance was lower than the accordance. A value of 1.0 lies just outwith the COR 95% confidence intervals (CI), indicating that the method was not quite as reproducible as repeatable. The diagnostic specificity, or percentage of correctly identified negative samples, was 96.1%, and 1.0 fell within the COR 95% CI, indicating that with identification of negative samples the method was as reproducible as it was repeatable. With the adenovirus / seawater method the diagnostic sensitivity was 89.3 %, and the concordance was lower than the accordance. Again, 1.0 lies just outwith the COR 95% confidence intervals (CI). The diagnostic specificity was 99.2 %, and 1.0 fell within the COR 95% CI. With the norovirus / freshwater method the diagnostic sensitivity was 91.4 %, and the concordance was lower than the accordance, 1.0 lying just outwith the COR 95% confidence intervals (CI). The diagnostic specificity was 96.1 %, and 1.0 fell within the COR 95% CI. With the norovirus / seawater method the diagnostic sensitivity was 91.7 %, and 1.0 fell within the COR 95% CI. The diagnostic specificity was 92.6 %, and 1.0 fell within the COR 95% CI.

4. Discussion

This study has shown clearly that it is possible to use relatively straightforward methods for the detection of two important enteric viruses in water samples across a range of geographical sites with varying degrees of pollution. The common occurrence of adenoviruses (36.4% of samples tested) reflected the intermittent shedding of HAdVs in the faeces by most adults.

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The difference in detection frequency may have been due to the greater dispersing and diluting power of the sea compared with that of the fresh waters. Alternatively, viruses may be less stable in marine waters due to the higher salt content, especially with higher temperatures (Hawley et al., 2008, Lo at al., 1976). The high frequency of G I detection by
5 two Laboratories suggests a higher level in the environment than was demonstrated by consideration of the rest of the data for this virus. It is known that detection of G I 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 G I viruses being under-represented among those found in clinical cases. It is unclear whether this
10 relates to our data as most of the G I isolates were found in only four sites. The frequency of G II norovirus detection was as expected.

The performance characteristics of the methods used for concentration and detection of HAdV and NoV in both fresh and marine water samples have been established. The percentage of correctly identified positive samples was around 90%, except for HAdV in
15 freshwater, which showed a sensitivity of 77 %, while the specificity of the methods was shown to be 93% or more, demonstrating that, where they may occur, there will be more false positive than false negative results. The sensitivity and specificity values compare well with those of some PCR-based methods for foodborne pathogen detection (Abdulmawjood et al., 2004; Malorny et al, 2004). The lower sensitivity value of the adenovirus / freshwater may
20 be due to the fact that the HAdV concentration in the seeded sample was lower than the NoV concentration used. This may also explain the higher COR values for the HAdV positive marine and freshwater samples. Furthermore, it should be noted that the samples used for the QC were not actually identical, whereas for the COR estimation this would be preferred. Each participant used the water from their own site(s), and this would differ from site to site
25 and from week to week. River water, particularly, will contain varying levels of material that may reduce the effectiveness of the concentration method and/or inhibit the molecular assays. Notwithstanding this, the results demonstrate that the methods used are robust, although currently, no criteria on lower limits of acceptability for robustness of methods for detection of viruses in water exist.

30 The theoretical limit of detection of the method reported here can be estimated. If an (RT-)PCR signal was obtained from an undiluted nucleic acid extract, and the assumption is made that the assay could detect one target molecule, this signifies that there was one virus

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equivalent in 10 μl nucleic acid extract. There were thus 10 virus equivalents in 100 ml nucleic acid extract, and on the assumption that this extract was obtained from 5 mL concentrate with no loss of target nucleic acid, this implies that there were 20 virus particles in the 10 mL concentrate. Assuming that the concentrate was derived from the original sample with no loss of virus, the conclusion is that a signal from the neat extract indicates that there were at least 20 virus particles in the 10 l water sample. If the extract had to be diluted to 10^{-1}, then there were 200 virus particles in the 10 l sample, and so on.

The amount of sewage discharged in the vicinity of many of the sites studied will affect the likelihood of human viruses being present in the water. Sewage input was not measured directly but the level of faecal indicators found reflects the contamination level. Viruses were found less often in sites where the sewage input was reckoned to be lower.

The influence of organic contaminants that occur naturally in water must not be underestimated. This is particularly true in fresh water and demonstrated in this study by the inhibition of the molecular assays. The use of the IACs in both NoV and HAdV PCRs was of significant benefit in guarding against false negative reactions. Reaction inhibition by substances in the sample is a well-known problem associated with analysis of environmental samples (e.g. da Silva et al., 2007). Dilution of the concentrate to produce a valid result, i.e. one where the IAC was detected, could also dilute any viruses present. In the current study the norovirus RT-PCR suffered some 5.5% of reactions failing to give a conclusive result (4.4% of fresh water samples and 7.7% of marine samples). Samples were tested at a higher dilution (up to 10^{-3}) to remove inhibition and achieve a positive IAC signal. Inhibition of the adenovirus PCR was also observed, though to a lesser extent, with PCR reactions of 0.9% of fresh water and 5.6% of marine water samples being inhibited, respectively. Samples from one inland major river site (Kew Bridge, UK) had often to be diluted up to 10^{-3} and consequently unexpectedly low numbers of samples positive for adenovirus (23%) were recorded. Subsequent tests with bovine serum albumin (BSA) in the PCR reaction suggest that routine use of this reagent may reduce enzymatic inhibition.

Integrated cell culture-PCR provided a method of determining the infectivity of adenoviruses, which was particularly useful since naturally-occurring virus strains do not always grow in cell culture with the same rapidity nor with the same evidence of cellular destruction. The enteric Ad40 and Ad41 viruses cannot be grown in most cell culture systems that support the growth of adenoviruses from the other subgroups, A549, HeLa, primary human amnion and

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primary human embryo kidney cells (Tiemessen et al., 1995). They have been shown to replicate in cell culture systems using Graham 293 cells, HEp-2 cells and HT-29 cells. Our data support these findings, because the presence of both Ad40 and Ad41 was shown by direct PCR, not in the cell culture-PCR assay using A549 cells (Ko et al., 2003, Tiemessen et al., 1995). Direct inoculation of cell cultures followed by observation over an extended period would not provide a good indication of infectivity and would not be in the interests of providing a rapid test. The finding that about 20% of fresh water samples and about 47% of marine water samples contained infectious adenovirus supports laboratory observations (e.g. Thurston-Enriquez, 2003) that these agents are environmentally robust.

The FIO levels encountered in this project exhibited a wide range from very 'clean' sites to those which would not pass Directive 76/160 regulatory values. The categorical association between adenovirus and the FIO regulatory parameters: i.e. *E. coli* and enterococci, was very statistically significant for fresh, but not marine waters studied. For norovirus a weaker association was observed between *E. coli* and norovirus in fresh waters but this was the only significant association between any FIO and this candidate viral parameter. This lack of association with FIOs, which have been proved to be predictive of health outcomes and used to design international standards for recreational waters is a cause for concern and suggests the need for further work before the viral parameters investigated here could be used as regulatory parameters in the absence of epidemiological data to provide an appropriate evidence-base for the policy community.

5. Conclusions

A comprehensive surveillance study of EU recreational waters was done through the 2006 bathing season. It may be concluded from the results that:

- almost 40% of bathing water samples in Europe were virus-positive entailing a possible public health risk from bathing;
- adenoviruses are more prevalent than noroviruses in both marine and fresh waters and appear to be a promising viral indicator for bathing waters;
- a single concentration method can be used to concentrate adenoviruses and noroviruses in fresh water recreational samples and a further single method can be used for marine waters;

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- concentration and detection methods may be used effectively even in polluted waters;
- adenoviruses are more prevalent than noroviruses in both marine and fresh waters;
- though the majority of sites returned frequencies of 0-25% positive, some were so polluted that >50% of samples contained one or both target viruses;
- adenoviruses remain infectious in the environment which may be true for other pathogenic viruses such as noroviruses.

The 'Virobathe' Group

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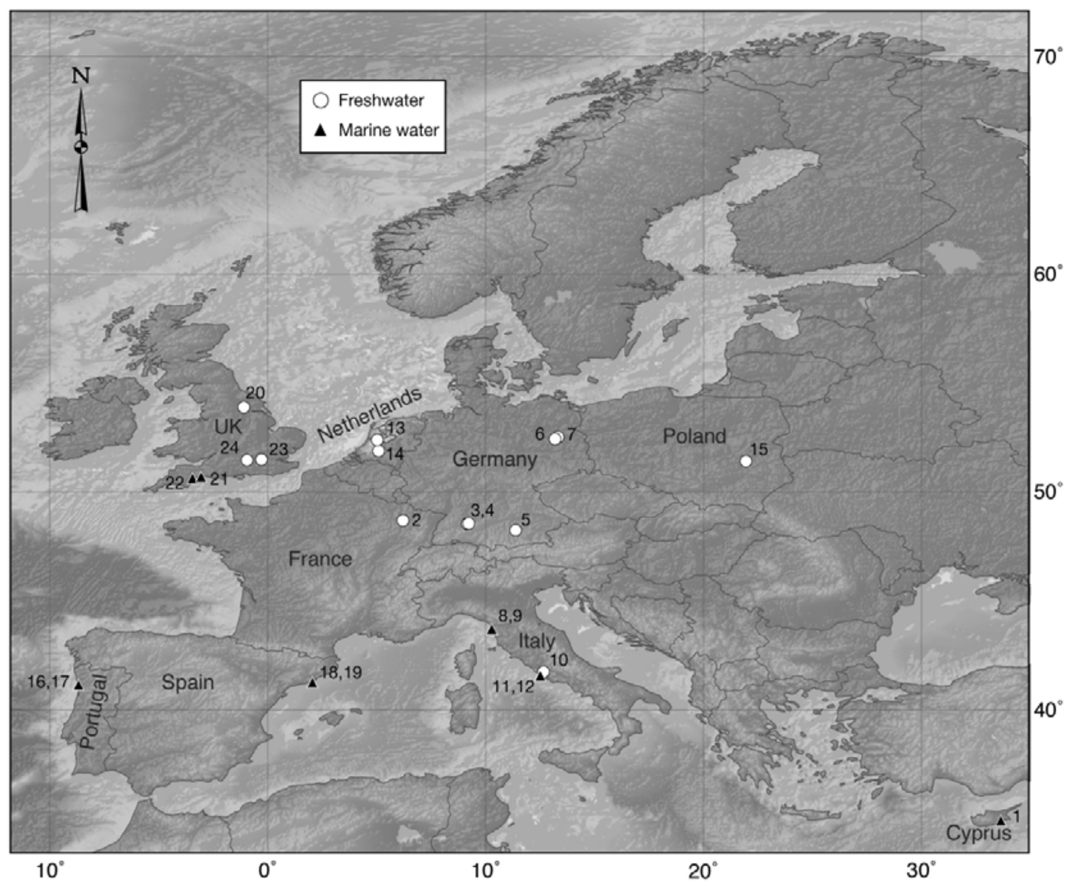

OR

Figure 1 Location of sampling sites

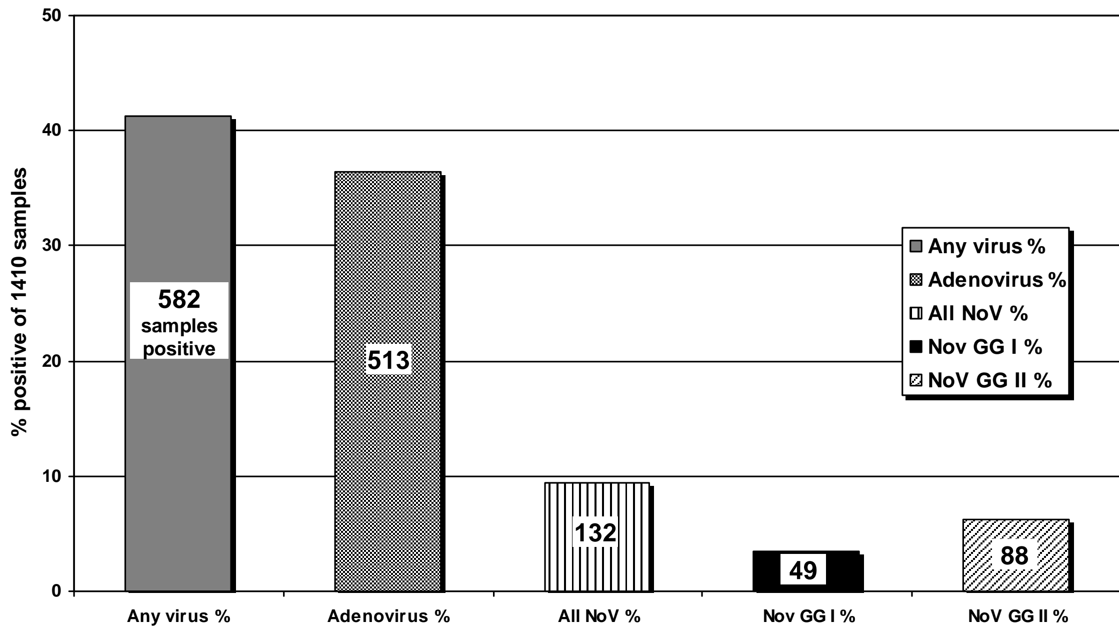

Fig 2: Summary of virus detection in all water types

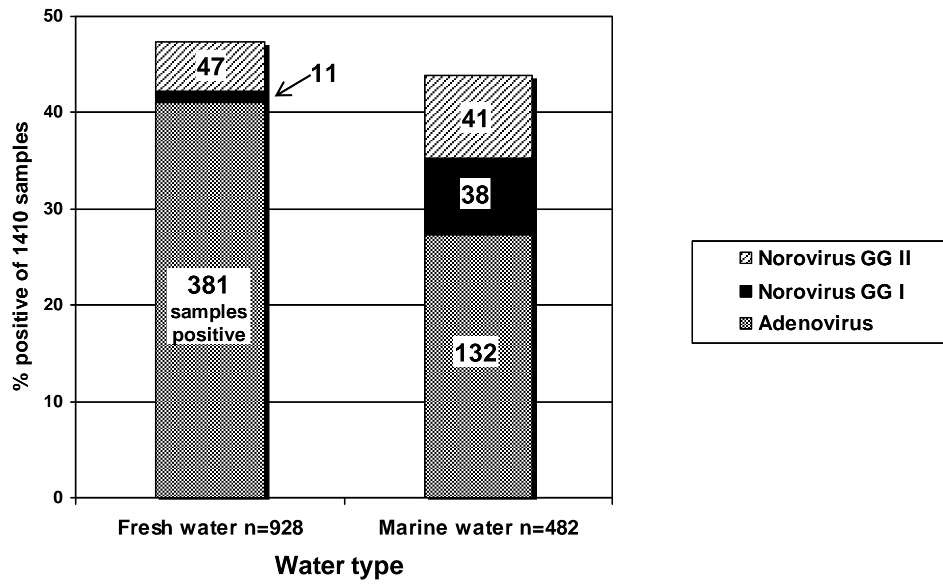

Figure 3: Virus detection by water type

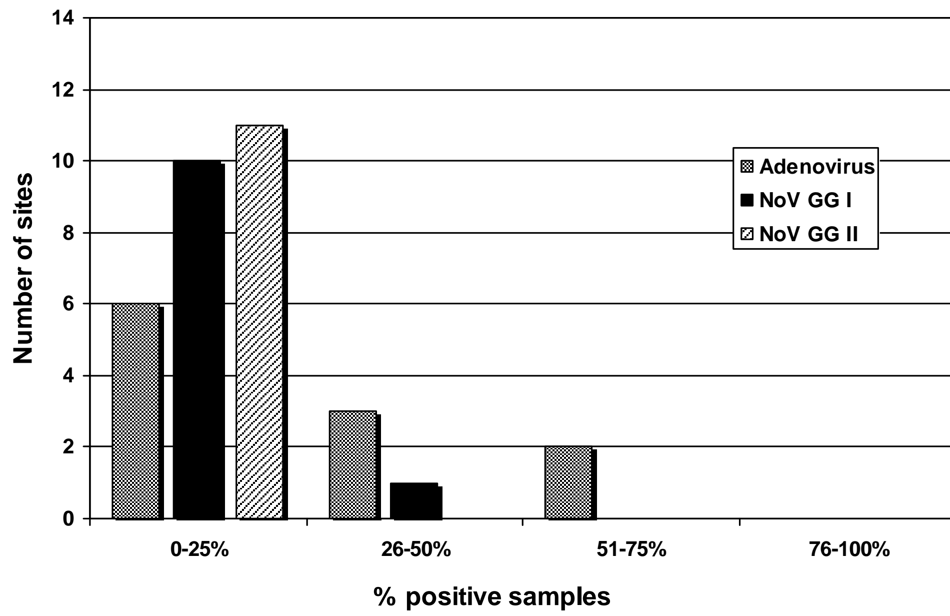

Figure 4: Distribution of virus-positive sites - marine

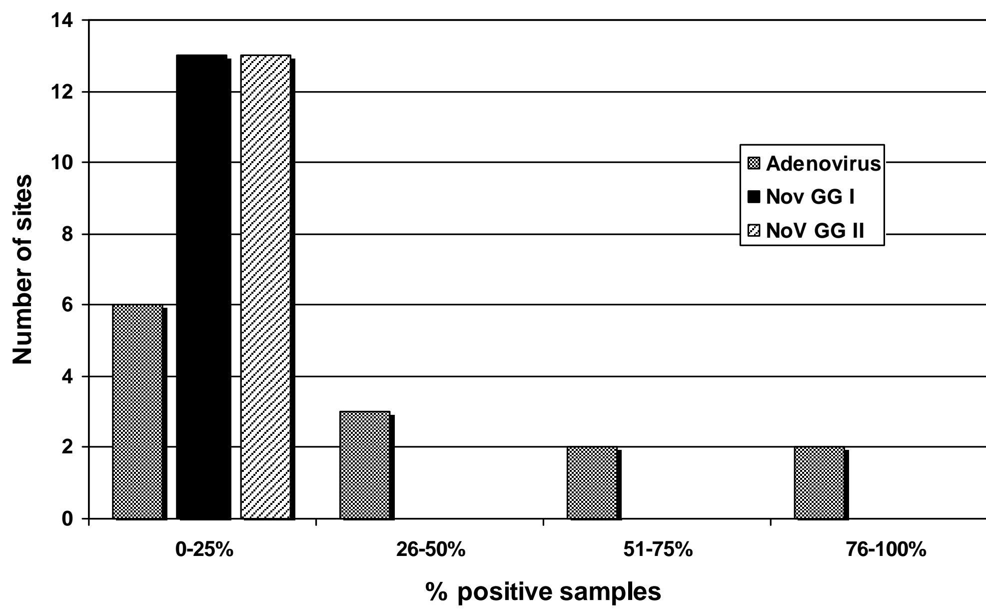

Figure 5: Distribution of virus-positive sites – fresh water

Table 1 - **Location of sampling sites**

	Country	Location	Site Name	Water Type
1	Cyprus	Larnaca	Larnaca Marina	Marine
2	France	Nancy	Tomblaine	Fresh
3	Germany	Baden-Württemberg	Neckar River	Fresh
4	Germany	Baden-Württemberg	Kirchentellinsfurt Lake	Fresh
5	Germany	Bavaria	Amper Grasslfing	Fresh
6	Germany	Berlin	Wannsee	Fresh
7	Germany	Berlin	Landwehrkanal	Fresh
8	Italy	Pisa	San Rossore	Marine
9	Italy	Pisa	Bocca d'Arno	Marine
10	Italy	Castel Gandolfo	Castel Gandolfo Lake	Fresh
11	Italy	Ardea (Rome)	Fosso dell'Incastro	Marine
12	Italy	Pomezia (Rome)	Rio Torto	Marine
13	Netherlands	Durgerdam	Kinselmeer	Fresh
14	Netherlands	Leerdam	Linge	Fresh
15	Poland	Pulawy	Vistula River	Fresh
16	Portugal	Porto	Molhe North	Marine
17	Portugal	Porto	Molhe South	Marine
18	Spain	Barcelona	Gavà	Marine
19	Spain	Barcelona	Gavà	Marine
20	UK	York	Naburn Lock	Fresh
21	UK	Devon	Axmouth Harbour	Marine
22	UK	Devon	River Kenn	Marine
23	UK	Kew (London)	River Thames	Fresh
24	UK	Reading	River Thames	Fresh

Table 2 - Variation in virus frequency

Water Type	% positive samples					
	HAdV		NoV GG I		NoV GG II	
	Highest	Lowest	Highest	Lowest	Highest	Lowest
Marine	55.0	0	30.0	0	16.3	0
Fresh	91.0	0	10.0	0	15.0	0

Table 3 - Range of virus frequencies

Water Type	Location	HAdV	NoV I	NoV II	EC	Qual	IE	Qual
Fresh	Kirchentellinsfurt	*0	0	0	^28	E	20	E
Fresh	Tomblaine	91	0	7.0	9656	P	683	P
Fresh	Amper-Grasslfing	80	0	0	13	E	8	E
Marine	Barcelona	0	0	0	15	E	15	E
Marine	Pomezia	55.0	30.0	10.0	602	P	169	G

* percentage of samples positive

^ GM (calculated by Mark Wyer, Period 2 Report p135/Table 3.5-3.6)

Table 4 - Virus infectivity

	T=0[1]	T=5[2]	Number of samples	% of those tested
Marine (51 tested)	-[3]	-	15	29
	-	+	24	47
	+	-	0	0
	+	+	12	24
Fresh (226 tested)	-	-	169	75
	-	+	46	20
	+	-	2	1
	+	+	9	4

[1] samples taken at time = zero (days); [2] samples taken at time = five days
[3] - = no cytopathic effect (c.p.e.) in cell culture, + = visible c.p.e.

Table 5 - Statistical evaluation of the adenovirus / freshwater detection method

Sample type	Sensitivity (%)	Specificity (%)	Accordance (%)	Concordance (%)	COR
Positive	77.2 (71.3 – 82.1)		73.9 (61.2 – 86.5)	63.5 (50.9 – 81.7)	1.63 (1.07 – 2.52)
Negative		96.1 (92.8 – 98.0)	93.0 (85.2 – 100)	92.5 (84.8 – 100)	1.08 (1.00 – 1.16)

Numbers in parentheses are the lower and upper 95 % confidence intervals.

Table 6 - Statistical evaluation of the adenovirus / seawater detection method

Sample type	Sensitivity (%)	Specificity (%)	Accordance (%)	Concordance (%)	COR
Positive	89.3 (82.5 – 93.6)		85.9 (68.9 – 94.9)	79.6 (66.1 – 92.7)	1.57 (1.01 – 2.29)
Negative		99.2 (95.5 – 99.9)	98.6 (97.4 - 100)	98.3 (94.6 - 100)	1.25 (0.97 – 1.44)

Table 7 -Statistical evaluation of the norovirus / freshwater detection method

Sample type	Sensitivity (%)	Specificity (%)	Accordance (%)	Concordance (%)	COR
Positive	91.4 (87.1 – 94.3)		86.2 (74.4 – 96.1)	83.9 (71.9 – 95.7)	1.2 (1.02 – 1.35)
Negative		96.1 (92.8 – 98)	92.9 (87 – 97.7)	92.5 (86.8 – 97.5)	1.06 (0.97 – 1.14)

Table 8 - Statistical evaluation of the norovirus / seawater detection method

Sample type	Sensitivity (%)	Specificity (%)	Accordance (%)	Concordance (%)	COR
Positive	91.7 (85.5 – 95.5)		85.3 (75.6 – 94.9)	84.6 (75.3 – 94.9)	1.05 (0.81 – 1.38)
Negative		92.6 (86.5 – 96.0)	88.0 (70.8 – 100)	85.7 (70.1 – 100)	1.22 (0.92 – 2.18)