Editorial Manager(tm) for Food and Environmental Virology
Manuscript Draft

Manuscript Number:

Title: Quantification of Human Adenoviruses in European Recreational Waters

Article Type: Original Research

Keywords: adenoviruses; quantitative real-time PCR; bathing waters; recreational waters; seawater; freshwater.

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Quantification of Human Adenoviruses in European Recreational Waters

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Keywords: adenoviruses, quantitative real-time PCR, bathing waters, recreational waters, seawater, freshwater.

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Abstract

The presence of human adenoviruses in recreational water might cause disease in the population upon exposure. Human adenoviruses detected by PCR could also serve as indicators of the virological water quality. In order to assess the applicability of human adenoviruses to the evaluation of the faecal contamination in European bathing waters, a real-time quantitative PCR assay was developed for the quantification of human adenoviruses in 132 samples collected from 24 different recreational marine and freshwater sites in nine European countries. Selected samples presenting positive nested-PCR results for human adenoviruses were analyzed using quantitative PCR and 80 samples from a total of 132 produced quantitative results with mean values of $3.2x10^2$ per 100 ml of water, human adenovirus 41 being the most prevalent serotype. Human adenoviruses were quantified in samples from all 15 surveillance laboratories. Statistical analysis showed no homogeneous linear relation between human adenoviruses and *E. coli*, intestinal enterococci or somatic coliphages concentrations in the tested samples when considering all the data together. Significant correlations between human adenoviruses and at least one of the other indicators were observed only when data from individual Laboratories were considered. The quantification of human adenoviruses may provide complementary information in relation to the use of bacterial standards in the control of water quality in bathing water.

Introduction

The presence of pathogenic microorganisms in faecally polluted recreational waters produces a perceived public health and economic problem, especially in countries which depend strongly on tourism. The European Bathing Water Directive (2006/7/EC) came into force in March 2006 to protect the health of the European bathers. The adequacy of using bacteria as indicators of the microbial water quality has been questioned since viruses and protozoan cysts have shown to be more resistant to treatment and disinfection processes commonly applied in sewage treatment plants (Tree et al. 2003). However, the new Directive does not include the analysis of viruses as one of the microbiological parameters listed. Article 14 of the Directive highlights a special interest on scientific, analytical and epidemiological developments relating to bathing water quality including those in relation to viruses, and encourages the report of these developments.

Human adenoviruses (HAdV) have been proposed as indicators of the presence of human fecal pathogens in the environment (Puig et al. 1998). HAdV have been demonstrated to be more prevalent than enteroviruses in water and shellfish (Pina et al. 1998), to be highly stable in the environment (Bofill-Mas et al. 2006) and highly resistant to disinfection treatments including UV radiation, especially adenoviruses 40 and 41 (Gerba et al. 2002; Thurston-Enriquez et al. 2003). Moreover, adenoviruses have been included in the U.S Environmental Protection Agency's contaminant candidate list (EPA CCL) and have been documented to be the second most significant cause of viral outbreaks in recreational waters (Sinclair et al. 2009).

Adenoviruses contain a double-stranded DNA genome of approximately 35,000 bp. They may be excreted in faeces for months or years following infection and may cause both enteric illness and respiratory and eye infections (Crabtree et al. 1997). Infection may be caused by consumption of contaminated water or food as well as by inhalation of aerosols during water recreation (Sinclair et al. 2009).

HAdV have previously been detected in environmental samples by PCR-based techniques (Pina et al. 1998, Bofill-Mas et al. 2006; Xagoraraki et al. 2007, Albinana-Gimenez et al. 2009). Occurrence of HAdV in river and coastal waters has been recently reviewed by Jiang (Jiang, 2006). Although quantitative real-time PCR (qPCR) methods for the quantification of some HAdV serotypes in diverse environmental samples have been recently described (Bofill-Mas et al. 2006; Choi and Jiang, 2005; Dong et al. 2009; Haramoto et al. 2007; He and Jiang, 2005; Jiang et al. 2005; Van Heerden et al. 2005a; Xagoraraki et al. 2007), to our knowledge, quantitative data on the occurrence of HAdV in European recreational waters has only been reported in one European country (Muscillo et al. 2008).

In this study, a real-time quantitative PCR assay (qPCR) was used for the quantification of HAdV in fresh and marine recreational waters of nine different European countries. The assay (Hernroth et al. 2002; Bofill et al. 2006) has previously demonstrated sensitive detection of the wide diversity of serotypes and has been used for the detection of HAdV in shellfish samples from divergent geographical areas (Formiga-Cruz et al. 2002) as well as for the monitoring of viral removal efficiency in a drinking-water treatment plant (Albinana-Gimenez et al. 2009), and for the detection and quantification of HAdV in different wastewater matrices (Bofill-Mas et al. 2006).

In this study, developed as part of the VIROBATHE project (a European Union Research Framework 6 funded project), a total of 132 fresh water and seawater samples collected from 24 different recreational sites in nine different European countries was analyzed for the presence of HAdV and the concentration of these viruses was estimated by qPCR. To evaluate the potential role of HAdV as an indicator of faecal contamination, the potential correlation between the HAdV genome copy numbers and bacterial and bacteriophage levels in these samples was also evaluated.

Materials and Methods

Environmental samples. In the bathing season of 2006, 10-L water samples were collected at approximately weekly intervals during the bathing season by 15 different laboratories from nine European countries (see Table 1 for a detailed list of countries), according to ISO 19458:2006. Samples were collected from 24 different sites representing typical seawater as well as freshwater bathing sites in the European Union. Samples were collected at least at six meters from the shore and 1 meter from the surface. Samples were processed within 24 hours after collection.

A 10-L sample of artificial seawater or freshwater was used as negative control of the concentration step and an extra sample, spiked with HAdV2 virus grown on A549 cells, was processed as a positive control of the concentration process.

Viral strains

To confirm the applicability of the assay, a collection of supernatants obtained from adenovirus-infected cell cultures from routine clinical analysis comprising representative serotypes of HAdV species A (31), B (3, 7, 7b, 35), C (1, 2, 6), D (37) and F (40, 41) were tested using the qPCR protocol.

During the study, the sensitivity of the qPCR assay applied in the different laboratories was tested by analyzing a commercial quantified suspension of HAdV5 DNA (ABI, Advanced Biotechnologies Incorporated. Columbia, Maryland, USA).

Bacteriological analysis *E. coli* (EC) and intestinal enterococci (IE) levels present in the samples were determined by Bio-Rad miniaturized methods using culture in liquid media (most probable number) for the detection and enumeration of *E. coli* (ISO 9308-3: 1998, water quality- Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 3:

Miniaturized method (Most Probable Number) for the detection and enumeration of *E.coli* in surface and waste water) and enterococci (ISO 7899-1: 1998. Water quality – Detection and enumeration of intestinal enterococci – Part 1: Miniaturized method (Most Probable Number) for surface and wastewater) in bathing, surface, and waste water (ISO 7899-1 and ISO 9308-3).

Bacteriophage analysis Somatic coliphage titres were determined following the double agar layer procedure as described in ISO 10705-2:2001, Detection and enumeration of bacteriophages. Part 2: Enumeration of somatic coliphages.

Concentration of viral particles from seawater samples. Recovery of viral particles from 10-L seawater samples was performed using either a procedure based on the use of cellulose nitrate membrane filters (Wallis and Melnick, 1967a; Wallis and Melnick, 1967b) and virus elution with glycine-skimmed milk buffer as described in (Bitton et al. 1979a; Bitton et al. 1967b) or a method based on a one-step concentration of viruses by direct flocculation with skimmed milk (Calgua et al. 2008).

Concentration of viral particles from freshwater samples. Recovery of viral particles from 10 liters of fresh water was performed by applying a procedure based on the use of glass wool columns and elution with glycine-beef extract buffer as described previously (Vilaginès et al. 1993).

Nucleic acid extraction. Nucleic acids were extracted from 5-ml sample concentrates using NucliSense® reagents (Biomeriéux, Boxtel, The Netherlands). For the seawater samples concentrated by the methodology described by Calgua et al. (2008), NucleoSpin RNA virus F

(Macherey & Nagel, Germany), was used for extraction of nucleic acids. Nucleic acids were frozen until further qPCR analysis.

Extracted viral nucleic acids were transported frozen when the qPCR assays were performed in a laboratory distant from the laboratory collecting and processing the samples.

Construction of qPCR standards. The DNA concentrations of plasmid pBR322 containing the HAdV 41 hexon sequence (kindly donated by Dr. Annika Allard, University of Umeå, Sweden) was estimated using Genequant pro (Amersham Biosciences). Ten µg of each DNA were linearized with BamHI, purified with the QIAquick PCR purification kit (QIAGEN, Inc.), quantified again and serially diluted such that 10 µl of the sample contained 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of DNA.

The stability of the standard DNA suspension was evaluated in 3 different eluents: DNA eluted with RNAse-free distilled water, Tris-EDTA, and the elution buffer provided in the NucliSens® kit from Biomerieux (Biomérieux, Boxtel, The Netherlands). Aliquots were kept at 4ºC and -80ºC for 3h and two weeks and variations on Ct values were analyzed by applying the qPCR as described. The stability of standard suspensions resuspended in TE buffer were also evaluated by repeated analysis after more than two weeks of storage at 4ºC and -80ºC.

qPCR assay for the quantification of HAdV DNA. Samples previously identified to be positive by nested-PCR (nPCR) analysis using the primers developed by Allard et al. (2001) were analyzed by qPCR. The assay applied in this study was been described by Hernroth et al. (2002) and is based in the amplification of the HAdV hexon gene. Amplifications were performed in a 25-µl reaction mixture containing 10 µl of DNA and 15 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems) containing 0.9 µM of each primer (AdF and AdR) and 0.225 µM of fluorogenic probe (AdP1) for HAdV detection. TaqMan® Universal

PCR Master Mix was supplied in a 2X concentration and contained AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, ROX as passive reference, optimized buffer components and AmpErase® uracil-N-glycosylase.

Following activation of the uracil-N-glycosylase (2 min, 50ºC) and activation of the AmpliTaq Gold for 10 min at 95ºC, 40 cycles (15 s at 95ºC and 1 min at 60ºC) were performed in the detection system currently used in every laboratory: Stratagene Mx3000P, ABI Sequence Detection System 7000 and LightCycler 480.

Neat and a ten-fold dilution of the DNA suspensions were run in duplicate (4 runs/sample) for analyzing environmental samples whereas each dilution of standard DNA suspensions (from 10^0 to 10^6) was run in triplicate. In all qPCRs the amount of DNA was defined as the mean of the data obtained. Standard precautions were applied in all assays, including separate areas for the different steps of the protocol and addition of non-template control (NTC) and non-amplification control (NAC) to each run. The presence of enzymatic inhibitors in the samples was evaluated by adding 10^4 GC of target DNA as an external control to the environmental samples assayed.

Sequence analysis of the PCR products obtained by nPCR. The amplicons obtained after nPCR assays of HAdV were purified using the QIAquick PCR purification kit (QIAGEN, Inc.). Purified DNA was directly sequenced with 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 nehex3deg and nehex4deg described by Allard et al. (2001) were used for sequencing at a concentration of 0.05 µM.

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/). 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/).

Statistical analysis In order to measure the correlation between HAdV genome copy numbers with the three other quantitative biological indicators (*E. coli* (EC), intestinal enterococci (IE), and somatic coliphages (SC) a synthetic approach based on a linear model was applied, simultaneously taking into account the possible effects of the water type and the laboratory. The variables were first transformed by using the log(x+1) function. With all the quantitative variables transformed, the model included the following sources of variation: (1) *type of water*, a fixed factor with two levels (marine or fresh), denoted by α in the equation below, (2) *laboratory*, a *nested* factor to water type, according to the Anova terminology, and denoted by β in the equation, and (3), the *interaction* between the *laboratory* and the *covariate* included in the model (EC, or IE or SC). This latter parameter is denoted by γ. We thus have three separate models including in each one a different covariate. For the three models, the following generic equation is applied:

$$y_{ijk} = \mu + \alpha_i + \beta_{j(i)} + \gamma_{j(i)} x_{ijk} + e_{ijk}$$

In the equation, y_{ijk} is the log transform of HAdV, x_{ijk} the log transform of the biological indicator considered (EC, or IE or SC) and e_{ijk} is the error term of the linear model. The sub indexes denote that the data correspond to the *k* sample in the *j* laboratory on the *i* water type. In the Anova literature, it is a classical model which allows testing on several groups the equality of the slopes of a linear relation between two variables.

Notice that if the Anova table shows the interaction γ term to be statistically significant, it must be interpreted that the slopes of the linear relation between *x* and *y* are different for some laboratories. If such is the case, the analysis must be conducted separately on each laboratory's data to estimate the linear relation between the variables. That is, the model must be reduced to the ordinary simple linear regression, splitting the full data set into several subsets corresponding to each laboratory:

$$y_{ijk} = \beta + \gamma x_{ijk} + e_{ijk}$$

All the statistical tests were computed using the statistical package Spss 15.0.1 (Spss Inc., Chicago, IL, USA).

Results

Specificity and sensitivity of the qPCR. The assay was selected for the quantification of HAdV in bathing waters because it was shown previously that the sensitivity of this assay was significantly higher than that obtained by other qPCR assays (Bofill-Mas et al. 2006).

The specificity of the assay was confirmed with a wide range of strains isolated by cell cultures from approximately 100 clinical samples. Serotypes of human adenovirus species A (adenovirus 31), B (3, 7, 7b, 35), C (1, 2, 6), D (37) and F (40, 41) were quantified by applying the HAdV qPCR assay here described. High concentrations of human polyomaviruses JCPyV and BKPyV, commonly present in urban sewage samples, were not detected by using the HAdV qPCR assay (data not shown).

The sensitivity of the assay was estimated to be 1-10 genome copies (GC) based on the data obtained in 20 different HAdV qPCR runs using synthetic plasmid DNA and the quantification of the commercial quantified suspension of HAdV5 DNA (ABI, Advanced Biotechnologies Incorporated. Columbia, Maryland, USA). A fluorescent signal was obtained in 90% of the runs when analyzing 10^0 GC according to spectrophotometrical measurements of standards. Thus, the sensitivity of the assay was confirmed to be between 1 to 10 GC for HAdV 5. The commercial standard was used as an intra laboratory control in all the laboratories performing qPCR analysis.

Stability of the DNA used as standard

To guard against the degradation of the qPCR standard DNA, stability was determined following storage for three hours at 4°C and -80°C in one of: molecular grade water, TE, or Biomérieux kit elution buffer. No significant differences were observed after storage of the DNA with the different eluents at -80ºC for 3h and 2 weeks. Ct values showed differences between different eluents and between different temperatures lower than 1 Ct. Moreover,

during the study aliquots of plasmids resuspended in TE were kept at 4ºC for more than 2 weeks and no differences in the Ct values were observed during qPCR reactions..

Virus recovery efficiency from water samples

HAdV2 virus preparations were used to spike positive control samples before concentration and nucleic acid extraction in order to quantify the recovery efficiency of the methods used.

The concentration method applied to determine the recovery of viruses from freshwater samples (glass wool concentration) showed an efficiency ranging from 6% to 81.5%. Two different concentration protocols had been applied to marine samples: a nitrocellulose negatively-charged membrane filter-based method showed highly variable recoveries ranging between 1.9% and 35.4% whereas one-step concentration by skimmed milk flocculation showed recoveries of 42.5-52.0% as described by Calgua et al. (2008).

Quantification of HAdV in recreational waters. A total of 132 nPCR HAdV positive seawater and freshwater samples were analyzed by the qPCR assay in different laboratories. The results obtained are summarized as mean values of all samples tested at each collection site (Table 1).

Eighty out of 132 samples (60.6%) tested positive with a mean value of $3.2x10^2$ GC/100ml of water. The percentage of positive samples was similar in both types of bathing water tested: 59.6% for marine and 61.3% for freshwater samples and mean values were $9.1x10^2$ ($3.3x10^1$-$2.0x10^3$) and $5.6x10^1$ GC/100 ml ($4.2x10^0$-$1.1x10^2$) of marine and freshwater, respectively.

Forty-seven samples were further typed by nPCR and sequenced: HAdV serotypes 12, 19, 31, 40 and 41 sequences were obtained with Ad41 being the serotype most commonly found.

Correlation of HAdV genome copy numbers with bacteria or/and bacteriophage titres

The relation between HAdV and the other microbiological parameters observed was highly variable and the statistical analysis of the data showed no significant correlation between the numbers of HAdV, bacterial standards and somatic coliphages analyzed.

For every covariate analyzed, table 2 shows strong evidence against equality of the slopes (p-values<0.05). There was also strong evidence against equality on the mean of HAdV detected by the laboratories (p-values<0.05), but not in the water type (p-values>0.05). The analysis of the residuals (not shown) confirms the adequacy of the log-transformation on the variables. Because the laboratory origin has significant effects on the slopes of the model for the three covariates (*E.coli*, IE, SC) the samples were analyzed separately. The linear regression analysis showed a significant linear relation between HAdV and the different variables tested in four laboratories (Table 3).

Discussion

In order to have rapid quantitative information on the level of fecal contamination in the recreational waters studied, a standardized quantitative real-time PCR assay was applied to the specific quantification of HAdV in recreational water samples. Cell culture assays, though providing quantitative information on infectivity have a very high cost and take several days to produce a result. Moreover, not all HAdV produce a distinct cytopathic effect in culture. The study presented was part of the VIROBATHE project that had as its main objective of evaluating the feasibility of trans-European analysis of viruses in recreational waters. The samples analyzed were selected on the basis of the results obtained by nPCR during a surveillance study including 15 European participant laboratories from nine different countries. The overall objective of the present study was to evaluate the applicability of the quantification of HAdV by qPCR as an index of the presence of human faecal contamination in European recreational waters.

HAdV were detected and quantified in both marine and freshwater collection sites including sites that, according to the European Bathing Water Directive (2006/7/EC), would be classified as bathing sites with good or excellent water quality, indicating that these are not free of the presence of HAdV DNA. However it should also be acknowledged that although HAdVs are known to be more stable than bacterial standards in the environment, especially in sea water and in most water treatments (Calgua et al. 2008; Albinana-Gimenez et al. 2009), the presence of viral DNA does not necessarily indicate the presence of infectious viruses. However, as part of the VIROBATHE project, the infectivity of HAdV was evaluated in some representative samples by ICC-PCR (Dong et al. 2009) and infectious HAdV were recovered from collection sites of laboratories 4, 10 and 13.

It is also important to notice that in some cases (for instance, in site 2), during the bathing period, considerably high concentrations of chlorine are added to the discharged wastewater

and this fact may influence the relation between bacterial standards that are more easily inactivated than HAdV, that are more resistant to chlorine disinfection. In addition chlorine-inactivated adenoviruses may produce positive qPCR results in the absence of detectable infectious particles. This could be the explanation for the significant negative correlation observed between HAdV and *E. coli* concentrations in the collection sites of Laboratory 2 (Table 3).

Standardization of the qPCR assay described was straightforward. Frozen nucleic acid extractions from overseas laboratories were transported without major problems during this study, and the DNA used as standard in the qPCR assays was also shown to retain high stability under different storage conditions.

The percentage of positive samples from the total number of samples collected in the study could not be evaluated, since qPCR was done on samples which had already tested positive by nPCR. However, as expected, high variability in the percentage of positivity has been observed in other studies (Van Heerden et al. 2003, 2005b; Miagostovich et al. 2008; Verheyen. 2009).

The methods applied in the study, represent low cost methods with acceptable values of recovery efficiencies, for marine samples concentrated by nitrocellulose membranes (1.9-35.4%) while alternative concentration methods by flocculation with skimmed milk showed more homogeneous recoveries (42.5-58%). Variable recoveries ranging from 6% to 81.5% for freshwater sample concentrated by using glass wool were obtained.

Not only some of the previously positive samples by nPCR were negative for qPCR but also some samples which had previously tested negative by nPCR produced positive results by qPCR (data not shown). Observed differences between nPCR and qPCR may be due to several factors such as small differences in sensitivity of qPCR and nPCR, different responses to enzymatic inhibition between qPCR and nPCR. qPCR because reduce the manipulation of the sample compared to nPCR and is less prone to PCR contaminants than conventional nPCR.

It should be also considered that when HAdV are present in concentrations near the limit of detection of the technique the analysis of different replicates may show different results.

Enzymatic inhibition has been observed by other authors when applying qPCR to environmental samples (e.g. Jiang, 2005). In our hands, enzymatic inhibition had been observed when applying the assay to samples with higher level of contamination (Bofill-Mas et al. 2006) and also in this study was observed in some of the sites studied in the undiluted sample. This inhibition is not inherent to qPCR as it has also been observed during this study when analyzing these samples by conventional nPCR techniques. Future efforts should be conducted to decrease enzymatic inhibition of samples to be tested by qPCR.

Different HAdV serotypes have been observed in positive qPCR, with HAdV 41 being the most commonly isolated serotype. The high prevalence of HAdV 41 in the samples studied is in accordance with what has been previously reported (Haramoto et al. 2007; Xagoraraki et al. 2007).

Statistical analysis evaluating potential correlations between the numbers of HAdV obtained in the study and the observed concentrations of IE, *E. coli* and SC in the tested samples showed no homogeneous linear relation between HAdV and the other variables when considering all the data.

The analysis of the linear model showed that the water type had no significant effects on the HAdV concentration measured. It shows also that the linear relation between HAdV and the other variables is not homogeneous across the laboratories and separate linear regressions show that only in 3 laboratories (4, 9 and 10) there is a significant correlation coefficient between HAdV and at least one of the covariates.

The qPCR methodology applied appears to be a technology feasible to standardise and to be repeatable in routine laboratories. The HAdV qPCR assay provides a quantitative estimation of the presence and sources of faecal contamination in the water and should be considered as a

molecular index providing complementary information for the control of water quality in bathing water.

Acknowledgments

The study here described was part of the Project VIROBATHE, a European Union Research Framework 6 funded project (Contract No. 513648) aimed at the rapid detection of viruses in recreational waters (www.virobathe.org). We thank all VIROBATHE participants for their collaboration providing us with samples to be analyzed in this study. The authors thank David Kay for his support as coordinator of VIROBATHE. During the development of this study Byron Calgua de León was a fellow of the MAEC-AECID, Spanish Government (Ministerio de Asuntos Exteriores y Cooperación).We thank Serveis Científico Tècnics of the University of Barcelona for the sequencing of PCR products.

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Figure legends

Figure 1. Comparison between mean value of IE and HAdV GC per 100 ml of water in the studied sites. Lines in bold indicate the maximum level of IE per each type of water (coastal and transitional or inland) required for good quality waters (based upon a 95-percentile evaluation) as established in the European Bathing Water Directive (2006/7/EC).

Figure 1

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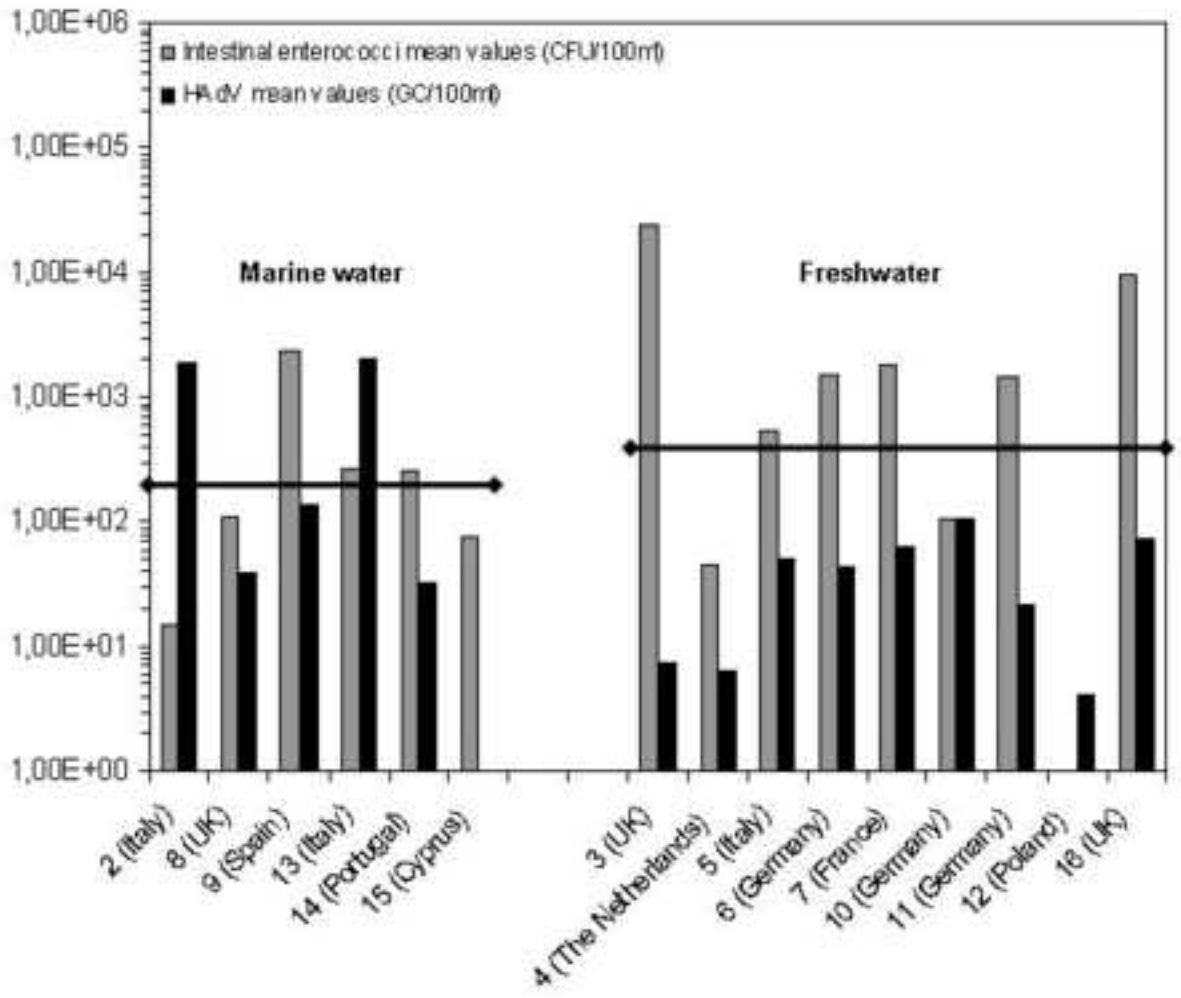

Table 1

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Table 1. Mean HAdV GC values observed per 100 ml of bathing water collected from different sites (countries). Mean values of *E. coli* , intestinal enterococci and somatic coliphages per 100 ml of water are also shown.

Samples	**Bacteria and phages**			**HAdV**		
Site number (Country)	***E. coli*** **CFU/100ml**	**Intestinal enterococci CFU/100ml**	**Somatic coliphages PFU/100ml**	**+qPCR/+nPCR[a]**	**HAdV range (GC/100ml)**	**HAdV mean value (GC/100ml)**
MARINE						
2 (Italy)	<15-30	<15	50-100	17/24	10.1-6482	1.8E+03
8 (UK)	400	108	1414	2/3	30.9-45.8	3.8E+01
9 (Spain)	2418	2418	4122	4/10	44.5-384	1.4E+02
13 (Italy)	473	263	31	7/9	10.2-13640	2.0E+03
14 (Portugal)	1310	252	NT[b]	3/10	2.3-94.8	3.3E+01
15 (Cyprus)	46	77	NT	0/1		
Total seawater				33/57		9.1E+02
FRESHWATER						
3 (UK)	2319	25008	750	4/8	4.8-213.3	7.4E+01
4 (The Netherlands)	179	46	430	3/14	43.2-89.8	6.4E+01
5 (Italy)	45	538	333	3/3	3-95.6	5.0E+01
6 (Germany)	9982	1511	2131	9/10	1.7-133.6	4.3E+01
7 (France)	12606	1791	1115	9/10	29.8-228.1	6.5E+01
10 (Germany)	3590	106	675	5/10	50.8-298.	1.1E+02
11 (Germany)	1392	1455	1733	3/6	0.6-63.2	2.2E+01
12 (Poland)	284	0	7.2	7/10	3.3-47.2	4.2E+00
16 (UK)	495585	9389	6788	4/4	13.1-202.8	7.4E+01
Total freshwater				47/75		5.6E+01
Total marine + freshwater				80/132		3.2E+02

[a] Number of positive QPCR samples out of total of analyzed nested PCR positive samples

[b] Non tested

Table 2. P-values corresponding to the different Anova tests. Each column corresponds to a model including one covariate.

Source of variation	*E.coli*	IE	SC
Water type	0.497	0.048	0.108
Laboratory	< 0.001	0.001	0.007
Covariate slope	0.001	0.015	0.031
R^2	0.486	0.432	0.417

Table 3. Laboratories showing significant linear relations between HAdV concentration and other microbiological parameters studied in the samples tested. Correlation coefficient and p-value (in parentheses) of the linearity test are shown.

Laboratory code	*E.coli*	IE	SC
Laboratory 2	-0.46 (0.037)		
Laboratory 4	0.62 (0.025)	0.65 (0.015)	0.66 (0.015)
Laboratory 9	0.72 (0.020)	0.69 (0.029)	0.72 (0.021)
Laboratory 10	0.74 (0.015)		