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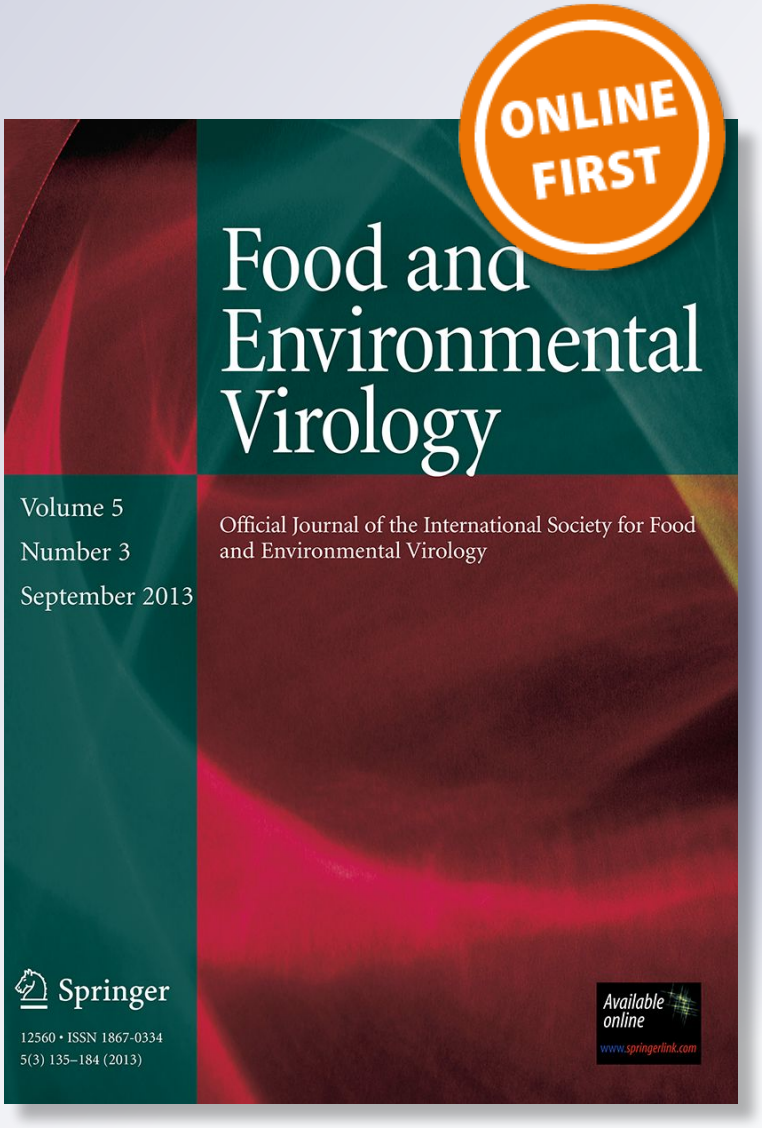

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ORIGINAL PAPER

Assessment of the Virological Quality of Marine and Running Surface Waters in NW Greece: A Case Study

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Abstract

The virological quality of surface marine and running water samples collected from Igoumenitsa gulf and Kalamas river (NW Greece) was assessed from October 2012 to September 2013. Sampling sites were exposed to different land and/or anthropogenic effects. Seawater samples were collected monthly from five sampling stations (new harbor, old harbor, wastewater treatment plant outlet, protected Natura area, Drepano beach). Viral targets included human adenoviruses (hAdVs), as index human viruses, while noroviruses (NoVs) and hepatitis A virus (HAV) were also studied. Kalamas river samples were collected seasonally, from three sampling stations (Soulopoulo, Dam, Sagiada-estuaries), while viral targets included also porcine adenoviruses (pAdVs) and bovine polyoma viruses (bPyVs), as additional index viruses. All water samples were analyzed for standard bacterial indicators, as well. Physicochemical and meteorological data were also collected. Based on the standard bacterial indices, both sea and river water samples did not exceed the limits set according to Directive 2006/7/EU. However, positive samples for hAdVs were found occasionally in all sampling sites in Igoumenitsa gulf (23.3%, 14/60) showing fecal contamination of human origin. Moreover, HAV was detected once, in the sampling site of the old port (at 510 GC/L). Most of the Kalamas water samples were found positive for hAdVs (58.3%, 7/12), while human noroviruses GI (NoVGI) (8.3%, 1/12) and GII (NoVGII) (16.7%, 2/12) were also detected. HAV, pAdVs, and bovine polyomaviruses (bPyVs) were not detected in any of the analyzed samples. No statistically significant correlations were found between classic bacterial indicators and viral targets, nor between viruses and meteorological data. Overall, the present study contributed to the collection of useful data for the biomonitoring of the region, and the assessment of the overall impact of anthropogenic activities. It provided also valuable information for the evaluation of the risk of waterborne viral infections and the protection of public health. It was the first virological study in the area and one of the few in Greece.

Keywords Virological quality · Human adenovirus · Norovirus · Hepatitis A virus · Bacterial indicators · Igoumenitsa Gulf · Kalamas river · Northwestern Greece

Introduction

Enteric viruses are shed in extremely high numbers in the feces and urine of infected individuals. They are usually transmitted via the fecal-oral route and primarily infect and replicate in the gastrointestinal tract of the host, being characterized by a low infective dose (Albinana-Gimenez et al. 2006; Kokkinos et al. 2010). These viruses are responsible for a large number of epidemics because of their presence in the aqueous environment or food (Sinclair et al. 2009; Carducci et al. 2009). Viral etiology is rarely identified, even though viruses are believed to cause a majority of waterborne marine illnesses (Griffin et al. 2003). Most of these viruses belong to the families Adenoviridae, Caliciviridae, Hepeviridae, Picornaviridae, and Reoviridae, and include major aetiological agents of mild diseases such as gastroenteritis as well as agents of more severe diseases such as meningitis and hepatitis (Rodríguez-Lázaro et al. 2012).

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Over the last decades, urban development and climate change are placing increasing pressure on coastal areas. Particularly in Greece, more than a half of the total population is estimated to live in coastal areas (CCISC 2011).

Igoumenitsa is the capital city of Thesprotia, located in the Northwestern part of Greece. The area hosts a port of international importance, as well as the wastewater treatment plant (WWTP) of the city. Fish farming and touristic activities are also recorded. A nature protection area of NATURA Network (GR 2120001) is located in the northwest part of the gulf. The last decades, Igoumenitsa gulf, has been under environmental pressure due to shipping, aquaculture, as well as agricultural, and tourism activities.

Previous studies performed in the same area have mainly focused on the prevalence of *Salmonella enterica* isolated from river and coastal waters (Economou et al. 2013), spatiotemporal dynamics of bacterioplankton communities (Meziti et al. 2015), benthic macrofauna (Karayanni et al. 2015), as well as on the measurement of physicochemical parameters in the sediment or the water column (Lelekis et al. 2001; Sylaios and Theocharis 2002), and the monitoring of priority pesticides (Lambropoulou et al. 2002) and pharmaceuticals (Nannou et al. 2015). Animal farming and agricultural polluting activities have been identified as the main problems for the Kalamas river basin management (Nannou et al. 2015). The driving-pressure-state-impact-response (DPSIR) model has previously used as an analytical framework for determining pressures and impacts under the Water Framework Directive 2000/60/EC (WFD), and is considered as an additional tool for both society and policy makers concerning water resources management. To support an integrated management in Kalamas River basin, the DPSIR scheme has been applied by Kagalou et al. (2012), verifying that the main driving forces, leading to pressures at the river basin, were the agriculture, the livestock, and the numerous point-pollution sources located at the catchment area, while river eutrophication was also reported (Kagalou et al. 2012).

According to previous study (Economou et al. 2013), and based on the standards set by the Council Directive 2006/7/EC, Kalamas was not safe for bathing. Kalamas water quality has deteriorated over the years, and different studies revealed higher human interference and microbial pollution in the lower part of the river (Lekka et al. 2004; Economou et al. 2013). Anthropogenic effects on bacterial diversity and function along Kalamas river-to-estuary gradient was studied by metagenomics (Meziti et al. 2016). Temporal differences of taxonomic and functional diversity were more pronounced than spatial ones. A highly dynamic ecosystem was revealed since $< 1\%$ of total taxa were shared among all samples. Even at tens of kilometers downstream from the city, microbial human gut signals were detectable over background freshwater and soil/run-off-related signals (Meziti et al. 2016).

The coastal waters of Plataria and Drepano were considered as bathing waters of excellent quality (Economou et al. 2013). As it was expected, one of the highest bacterial counts in marine water was observed at the Igoumenitsa port, where water is polluted by urban and rural effluents (Economou et al. 2013). No *Campylobacter* spp., *E. coli* O157:H7, *Cryptosporidium*, and *Giardia* were isolated from Kalamas river and seawater samples (Karanis et al. 2002; Economou et al. 2013).

Besides bacteria, the presence of anthropogenic viruses in marine waters has been, and will continue to be, an important public health issue. Almost 40% of bathing water samples in Europe have been previously recorded as virus genome positive implying a possible public health risk from bathing. Notably adenoviruses appear more prevalent in marine waters than noroviruses and have been suggested as a promising viral indicator for bathing water quality (Wyn-Jones et al. 2011). Enteric viruses in water may originate from runoff of animal manure, from discharges of raw or treated sewage, or directly from humans or animals (Griffin et al. 2003). HAdVs are associated with sporadic cases and occasional outbreaks of gastroenteritis, and have been extensively proposed as indicators of human fecal contamination in water. (Pina et al. 1998; Bofill-Mas et al. 2006, 2010; Kokkinos et al. 2011; Rodríguez-Lázaro et al. 2012). NoVs have been documented as waterborne pathogens, and numerous norovirus outbreaks have originated from sewage-polluted recreational water (Sinclair et al. 2009; Wyn-Jones et al. 2011; Rodríguez-Lázaro et al. 2012). HAV is the main cause of acute hepatitis worldwide (Costafreda et al. 2006). It has been associated with many outbreaks linked to sewage contamination of recreational waters, while data from many studies reflect its persistent circulation in marine environments and the Mediterranean region (Griffin et al. 2003; Vantarakis et al. 2009; Rodríguez-Lázaro et al. 2012). PAdVs and bPyVs have been used as index viruses for fecal contamination of animal origin (Hundesa et al. 2009, 2010).

In the present study, we aimed at the assessment of the overall impact of different anthropogenic activities by investigating the virological quality of sea and surface water samples collected from Igoumenitsa gulf and Kalamas river, respectively. To the best of our knowledge, it is the first time that virological data are collected in the study area. These data are expected to provide useful information for the risk assessment of waterborne infections and the protection of Public Health.

Materials and Methods

Sampling Area

The Igoumenitsa gulf is situated in the southern end of the Corfu channel and approximately 11 km to the south from

the Kalamas river outfall in the Ionian Sea. It receives rural and agricultural effluents and harbors the Igoumenitsa city (population 14,710) (Fig. 1). Igoumenitsa gulf is a semi-enclosed gulf impacted by different anthropogenic activities. Igoumenitsa port is one of the main sea entrances to Greece for passengers and goods.

The old port accommodates small fishing boats. In its southern side, the new ferry port for national routes is located. The new port has a land zone of 21,000 m^2, and 12 mooring positions are able to service up to seven ships at the same time (http://www.olig.gr). Aquaculture facilities are also located in Igoumenitsa gulf, with production capacities of 600 tons of fish species such as bream (*Sparus aurata*), and bass (*Dicentrarchus labrax*) (http://www.fishfarms.gr/meli).

The WWTP of Igoumenitsa (code: GR21200101) was inaugurated in 2003 and is located at the entrance of the gulf. It is a secondary treatment plant, with a separated sewer, treating urban sewage and cesspool waste. The mean (annual mean value) volume of influent wastewater is 3200 m^3/d of which 3000 m^3/d is sewage (population equivalent 14,800) and 200 m^3/d is cesspool waste (population equivalent 3350). The treated effluents, which are disinfected by chlorination, are disposed to the Ionian Sea (water receiver code: GR2120010120).

The gulf of Igoumenitsa neighbors also to a Natura 2000 protected area of 8531 ha, the Kalamas River Delta. The area is important both at national (nature reserve, N.1650/86, Article 19 paragr. 2) and European level (has designated a Site of Community Importance code GR 2120001 and Special Protection Area for birds with code GR 2120005), because of its rich biodiversity and its geographical position which renders it an integral part of western migration route of birds.

A peninsula with an area of 32 acres is located at the entrance of the gulf. It is surrounded by sandy beaches hosting swimming and other recreational activities, and it is almost completely covered by tall eucalyptus trees.

Kalamas River has a length of 115 km, a mean annual flow of 57 m^3 s^{-1}, a mean depth of 2.7 m, and a catchment area of 1831 km^2 from which 43.6 km^2 is irrigated (Fig. 1). Its main uses are irrigation and aquaculture, while it is also used for recreational activities such as rafting, boating, fishing, and swimming. A tunnel and ditch were constructed in 1960 to drain the overflow of the eutrophic Lake Pamvotis (in Ioannina city) to Kalamas and from 1992, the treated discharges of the Ioannina city sewage plant are released into the basin of this river (Economou et al. 2013). Kalamas estuary is located north of Igoumenitsa gulf and the region around it has been included in NATURA 2000 network.

Sampling Strategy

Seawater sampling was performed according to the standard bottle sampling depth (2 m) for surface waters in the open ocean but also in coastalwaters (to avoid high UV, neuston, sediment resuspension in shallow waters etc). Seawater sampling (10 L) was performed monthly between October 2012 and September 2013 in five sites (S) located in different areas of the gulf (Fig. 1): the old harbor (S1), the new harbor (S2), the WWTP (S3), the protected NATURA area (S4), and the outer area (S5) near Drepano beach. Kalamas river samples (10 L) were collected seasonally (November 2012, February, May, July 2013), from three sampling sites: Soulopoulo (exit of Lapsista ditch to Kalamas river, K1), Dam (K2), and Sagiada (estuaries north of Igoumenitsa gulf, K3).

Physicochemical Parameters

During sampling, physicochemical parameters were measured: air and water temperature, pH, and chlorophylls. Photosynthetic pigments were determined spectrophotometrically as described in Meziti et al. (2015).

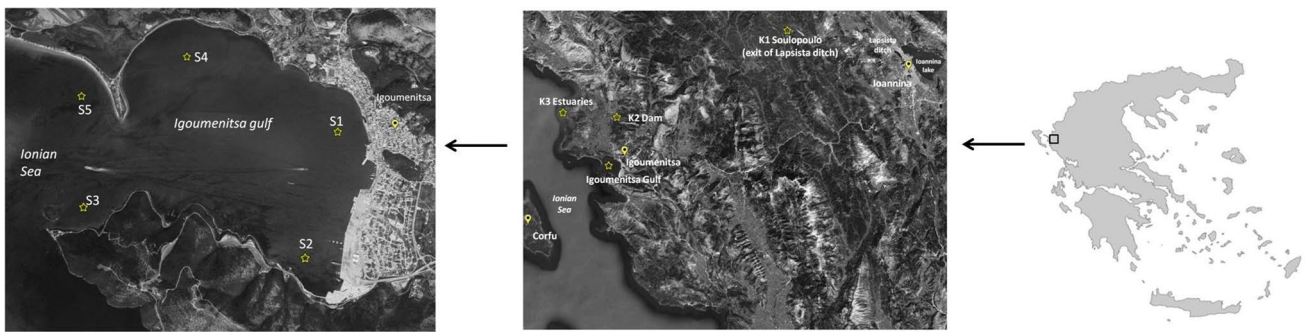

Fig. 1 Sampling sites at Igoumenitsa Gulf (S1–S5) and Kalamas river (K1–K3)

Microbiological Analysis of Sea and Surface Water Samples

Bacterial Indicators

Analysis for the classic bacterial indicators of sea and surface water samples was performed according to ISO methods. Samples were subjected to standard analysis for coliform bacteria and *E. coli* (ISO 9308-01: 2000), intestinal enterococci (ISO 7899-02: 2000), and total count at 22 °C, and at 37 °C (ISO 6222: 1999).

Virological Analysis

Ten liters (10 l) of seawater and/or river water samples was collected into aseptic containers and then transported to the laboratory. For viral detection, a concentration procedure was applied (Skimmed Milk Flocculation, SMF) (Calgua et al. 2008). Briefly, once the samples were conditioned, a pre-flocculated 1% (w/v) skimmed milk solution (PSM) was prepared by dissolving 10 g skimmed milk powder (Difco, Detroit, MI, USA) in 1 L of artificial seawater at pH 3.5 (Sigma-Aldrich Chemie GMBH, Steinheim, Germany). The sample was then acidified to pH 3.5 by adding HCl 1 N, and the PSM was added to each of the previously conditioned samples which were stirred for 8 h at room temperature, while the flocs were allowed to form sediment by gravity for another 8 h. Then, the supernatant was carefully removed using a vacuum pump without disturbing the sediment, and the final volume of about 500 mL containing the sediment was transferred to a centrifuge tube and centrifuged at 7000×*g* for 30 min at 12 °C. The supernatant was carefully removed and the pellet dissolved in phosphate buffer at pH 7.5, at a ratio of 1 mL of phosphate buffer per 1 L of concentrated sample. The viral concentrate was stored at − 80 °C. For viral nucleic acids extraction, a commercial kit (QIAamp Viral RNA Mini Kit, Qiagen) was used. Viral molecular detection was based on TaqMan assays previously described by Hernroth et al. (2002) for hAdV, Svraka et al. (2007) for NoV GI, da Silva et al. (2007) for NoV GII, and Costafreda et al. (2006) for HAV. Kalamas river samples were additionally analyzed for porcine adenoviruses and bovine polyoma index viruses. Molecular detection was also based on previously described TaqMan assays by Hundesa et al. (2009) for pAdV, and Hundesa et al. (2010) for bPyV. Primers, probes, and amplification conditions of the viral targets of the study are summarized in Table 1. Ten-µL sample of nucleic acid extract was added to make a final reaction volume of 20 µL. RNA Ultrasense reaction mix (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems) were used for the detection of RNA and DNA viral targets, respectively. The viral molecular detection was performed on a Stratagene MX3005p platform. The analysis method is accredited by ISO 17025.

Table 1 Primers, probes, and amplification conditions of the viral targets of the study

Target	Name	Sequence (5′–3′)	Amplification conditions	References
HAdV	AdF	CWTACATGCACATCKCSGG	1 cycle: 2 min 50 °C	Hernroth et al. (2002)
	AdR	CRCGGGCRAAYTGCACCAG	1 cycle: 10 min 95 °C	
	AdP1	6FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ	45 cycles: 15 s 95 °C + 1 min 60 °C	
HAV	HAV68	TCACCGCCGTTTGCCTAG	1 cycle: 15 min 50 °C	Costafreda et al. (2006)
	HAV240	GGAGAGCCCTGGAAGAAAG	1 cycle: 2 min 95 °C	
	HAV150(-)	6FAM-CCTGAACCTGCAGGAATTAA-MGBNFQ	45 cycles: 15 s 95 °C + 1 min 60 °C	
NoVGI	QNIF4	CGCTGGATGCGNTTCCAT	1 cycle: 15 min 50 °C	Svraka et al. (2007)
	NV1LCR	CCTTAGACGCCATCATCATTTAC	1 cycle: 2 min 95 °C	
	NV1LCpr	6FAM-TGGACAGGAGAYCGCRATCT-BHQ	45 cycles: 15 s 95 °C + 1 min 60 °C	
NoVGII	QNIF2d	ATGTTCAGRTGGATGAGRTTCTCWGA	1 cycle: 15 min 50 °C	da Silva et al. (2007)
	COG2R	TCGACGCCATCTTCATTCACA	1 cycle: 2 min 95 °C	
	QNIFS	6FAM-AGCACGTGGGAGGGCGATCG-BHQ	45 cycles: 15 s 95 °C + 1 min 60 °C	
pAdV	Q-PAdV-F	AACGGCCGCTACTGCAAG	1 cycle: 10 min 95 °C	Hundesa et al. (2009)
	Q-PAdV-R	AGCAGCAGGCTCTTGAGG	45 cycles	
	Q-PAdV-P	6FAM-CACATCCAGGTGCCGC-BHQ	15 s 95 °C + 1 min 60 °C	
bPyV	QB-F1-1	CTAGATCCTACCCTCAAGGGAAT	1 cycle: 10 min 95 °C	Hundesa et al. (2010)
	QB-R1-1	TTACTTGGATCTGGACACCAAC	45 cycles	
	QB-P1-2	6FAM-GACAAAGATGGTGTGTATCCTGTTGA-BHQ	15 s 95 °C + 1 min 60 °C	

Quantitation

Quantitation of the target viruses was performed as described previously (Kokkinos et al. 2012). Quantitation methods were based on the Standard Operating Procedures (SOPs) developed by VITAL (Integrated monitoring and control of foodborne viruses in European food supply chains) (FP-7, KBBE) project. Quantitation was performed by the most probable number approach. The nucleic acid extract was assayed neat, and in 10^{-1} dilution, and two replicate assays were performed for each concentration. Synthetic multiple-target RNA and DNA oligonucleotides were constructed for use as quantification standards for nucleic acid amplification assays, overcoming the problems related to the difficulty of obtaining practical quantities of viral RNA and DNA from the target viruses (D'Agostino et al. 2011; Martínez-Martínez et al. 2011).

Meteorological Monitoring

Meteorological data (temperature, rain) were collected from the Meteodata-base of the National Observatory of Athens (NOA), through the Meteo Search publicly accessible website (http://meteosearch.meteo.gr/). The network of automatic stations consists of stations type Davis, which measures all basic meteorological parameters, i.e., pressure, temperature, humidity, rainfall, direction, and wind strength. The meteorological data from Igoumenitsa were collected from the station "Igoumenitsa LG43," which is located at Mavroudi location, at a height of 77 m. Ultraviolet (UV) (310 nm-UVB, 380 nm-UVA mW/m^2 nm), ozone monitoring instrument daily satellite data (AURA/OMI) were also provided by the NOA, after request.

Statistical Analysis

Descriptive statistics were performed and summarized for each parameter of physicochemical characterization and meteorological monitoring among the different sampling stations. Shapiro–Wilk was used for performing tests of normality for the quantitative parameters (p value < 0.5). Moreover, it was investigated if any possible statistical correlations existed for all groups of variables (meteorological, physicochemical, and microbiological—virus, bacterial indicators) with the implementation of Spearman's test. The statistical software that was used for the statistical analysis was SPSS 21.0 (IBM, USA).

Results

Physicochemical Characterization and Meteorological Monitoring

Physicochemical characterization data along with meteorological monitoring data are summarized in Table 2.

Igoumenitsa Gulf

Mean ± standard deviation, minimum, and maximum values are presented per sampling site (S1–S5) (Table 2). Seawater temperature ranged between 11.8 and 28 °C. According to chlorophyll-a values, the area presented a late winter-early spring as well as an autumn phytoplankton bloom. In general, chlorophyll-a concentration presented similar temporal variations in the different sampling sites. Maxima (5.8 μg L^{-1}) occurred at the new port (S2) in September.

Kalamas River

Data have been summarized per sampling site (K1–K3) (Table 2). River water temperature ranged between 10 and 22 °C. Chlorophyll-a maximum values occurred in K1 sampling site in February (2.1 μg L^{-1}) and May (1.7 μg L^{-1}).

Microbiological Analysis of Seawater Samples

Bacterial Indicators

Descriptive statistics for bacterial indicators data are summarized in Table 3.

Igoumenitsa Gulf Based on the classic bacterial indices and according to Directive 2006/7/EU (incorporated to the Greek Law by the Joint Ministerial Decision 8600/416/E103, Government Gazette 356/26-2-2009), the analyzed seawater samples were of exceptional quality, since the highest counts for *E. coli* and enterococci (S1, January) did not exceed the limits of 250 and 100 cfu/100 mL, respectively.

Kalamas River The highest counts for *E. coli* and enterococci in river water samples did not exceed the "sufficient" limits of 900 and 330 cfu/100 mL, respectively (which have been set for inland waters according to Council Directive 2006/7/EC imposing new criteria for the characterization of water intended for recreational use).

Virological Analysis

Virological data per sampling site and virus type are summarized in Table 4.

Igoumenitsa Gulf Positive samples for hAdVs (14/60, 23.33%) were found occasionally in all sampling sites (Fig. 1). However, the majority of seawater samples (46/60, 76.66%) were found negative to hAdVs. The highest hAdVs concentrations were found at S5 in October (2899 GCs/L) and August (1582 GCs/L) and at S3 in November (1006 GCs/L). Human NoVGI/GII were not detected during the

Table 2 Summarized physicochemical and meteorological monitoring data for Igoumenitsa Gulf and Kalamas river

	Tair	Tw	UVA	UVB	pH	Chla
S1						
Mean ± SD	21.6 ± 6.5	20.5 ± 5	473.5 ± 197.2	57.8 ± 33.4	6.9 ± 0.3	8.3 ± 1.5
Min	11	13.4	156.6	14.1	6.2	5.8
Max	30	28	781.7	117.5	7.5	10.6
S2						
Mean ± SD	23.1 ± 6.5	20 ± 4.9	473.5 ± 197.2	57.8 ± 33.4	7 ± 0.3	8.1 ± 1.5
Min	11	13.6	156.6	14.1	6.5	5.7
Max	30	27.2	781.7	117.5	7.5	10.7
S3						
Mean ± SD	21.7 ± 6.7	20.2 ± 4.9	473.5 ± 197.2	57.8 ± 33.4	6.9 ± 0.4	7.7 ± 1.5
Min	11	13.8	156.6	14.1	6.5	6.0
Max	29	27.2	781.7	117.5	7.5	10.5
S4						
Mean ± SD	21.5 ± 6.4	19.8 ± 5.4	473.5 ± 197.2	57.8 ± 33.4	6.8 ± 0.3	7.7 ± 1.5
Min	11	11.8	156.6	14.1	6.5	5.3
Max	29	27	781.7	117.5	7.5	9.7
S5						
Mean ± SD	23.8 ± 5.6	19.7 ± 4.8	473.5 ± 197.2	57.8 ± 33.4	6.9 ± 0.4	7.7 ± 1.5
Min	13.5	13.2	156.6	14.1	6.5	5.3
Max	30	27.1	782	118	7.5	9.7
K1						
Mean ± SD	20.5 ± 7.9	14.8 ± 2.6	540.9 ± 253	66.3 ± 40.2	6.6 ± 0.3	1.1 ± 0.9
Min	11	11	204.6	18.4	6.7	0.68
Max	30	17	742.6	104.4	7	2.07
K2						
Mean ± SD	19.5 ± 6	16.3 ± 4.8	449.1 ± 227.3	54.6 ± 36	6.3 ± 0.3	0.6 ± 0.3
Min	12	10	204.6	36	5.8	0.33
Max	26	20	742.6	104.4	6.5	0.98
K3						
Mean ± SD	21.6 ± 7	15.9 ± 4.9	449.1 ± 227.3	449.1 ± 227.3	6.6 ± 0.3	0.6 ± 0.4
Min	11.4	10.5	204.6	54.6	6.5	0.01
Max	27	22	742.6	36	7	0.94

S1–S5 Sampling sites at Igoumenitsa Gulf, *K1–K3* sampling sites at Kalamas river, *Tair* (°C) air temperature, *Tw* (°C) water temperature, *UVA* (380 nm mW/m^2/nm): ultraviolet A, *UVB* (310 nm mW/m^2/nm): ultraviolet B, *Chla* (µg/L): chlorophyll-a

Table 3 Concentration (cfu/100 mL) of *E. coli*, enterococci and total coliforms in seawater (IG1–IG5) and river (K1-K3) water samples

Sampling point	*E. coli*		Enterococci		Total Coliforms	
	AVG	SD	AVG	SD	AVG	SD
IG1	7.1	23	4.8	14.1	22.7	56.9
IG2	0.8	1.2	0.3	0.6	7.4	12.2
IG3	0.4	0.8	1.2	3.5	6.5	9.3
IG4	1.2	2.2	1.8	4.9	13.4	19.9
IG5	0.3	0.9	0.6	1.2	5.8	8.6
K1	140	147.6	80	53.1	251.5	137.7
K2	83.8	124.5	21.3	16.6	211.8	104.2
K3	304	416.2	25.3	18.3	236	151.8

AVG average; *SD* standard deviation

Average and standard deviation over the 12-month study period (IG, $n = 12$; KAL, $n = 4$)

study, while HAV was detected only once at S1 in June (510 GC/L).

Kalamas river Most of the river water samples were found positive for hAdVs (7 out of 12 samples). The highest hAdVs concentrations (244 GCs/L) were found at K2 in November (244 GCs/L) and K3 in May (166 GCs/L). Human NoVGI were also detected at K2 during November (< 50 GCs/L), while human NoVGII were also found at K2 during November (< 50 GCs/L) and at K3 during November (< 50 GCs/L). HAV, pAdVs, and bPyVs were not detected in any of the analyzed samples.

Statistical Analysis

The statistical analysis revealed statistically significant correlations between bacterial indices (*E. coli*, total coliforms, enterococcus) and physicochemical parameters (temperature, pH). No statistically significant correlations were found between classic bacterial indicators and viral targets, nor between viruses and meteorological data (Table 5).

Discussion

Based on the classic bacterial indicators, the analyzed seawater samples were of exceptional quality, since the highest counts for *E. coli* and enterococci were below the limit values set by the Directive 2006/7/EU. Interestingly, a parallel study investigating bacterial community structure in the water column using next generation sequencing techniques did not reveal the presence of pathogenic bacteria in the area (Meziti et al. 2015). Similarly, no *Campylobacter* spp., *E. coli O157:H7, Cryptosporidium*, and *Giardia* were isolated from marine water samples collected from the study area by Economou et al. (2013).

In accordance to numerous previous studies, the present study did not reveal any statistically significant correlation between standard bacterial water quality indicators and the human viruses (Griffin et al. 2003; Kokkinos et al. 2010, 2011; Korajkic et al. 2011; Staley et al. 2012; Rodríguez-Lázaro et al. 2012; Lin and Ganesh 2013). Overall, although according to standard bacterial indicators, water quality was very good, 23.3% of the samples were found positive to hAdVs; hence, our results should be considered as indicative of human fecal contamination in the area (Bofill-Mas et al. 2006, 2010; Wyn-Jones et al. 2011; Rodríguez-Lázaro 2012). It should be noted that hAdVs are common causative agents of gastroenteritis outbreaks and are believed to

Table 4 Summarized results of the virological data per sampling site, and virus type

Sampling site	hAdV	HAV	NoV GI	NoV GII	pAdV	bPyV
S1	3/12 (25%)	1/12 (8.3%)	0/12	0/12	nd	nd
S2	3/12 (25%)	0/12	0/12	0/12	nd	nd
S3	3/12 (25%)	0/12	0/12	0/12	nd	nd
S4	2/12 (16.7%)	0/12	0/12	0/12	nd	nd
S5	2/12 (16.7%)	0/12	0/12	0/12	nd	nd
K1	1/4 (25%)	0/4	0/4	0/4	0/4	0/4
K2	3/4 (75%)	0/4	1/4 (25%)	1/4 (25%)	0/4	0/4
K3	3/4 (75%)	0/4	0/4	1/4 (25%)	0/4	0/4

Number of positives/number tested; *nd* no data, *S1–S5* Sampling sites at Igoumenitsa Gulf, *K1–K3* sampling sites at Kalamas river

Table 5 Statistical correlations among indicator bacteria, human adenoviruses, and physicochemical parameters (significant results are in bold)

	hadV	Total Coliforms	*E. coli*	Enterococcus	Chla	Salinity	Temperature	pH
hadV	1.00							
Total Coliforms	0.15	1.00	**0.76**	**0.71**	0.02	−0.06	**−0.78**	**−0.46**
E. coli	0.04	**0.76**	1.00	**0.52**	0.08	−0.17	**−0.58**	−0.45
Enterococcus	0.12	**0.71**	**0.52**	1.00	0.1	−0.37	**−0.5**	**−0.54**
Chla	−0.11	0.02	0.08	0.1	1.00	−0.14	0.03	−0.09
Salinity	−0.03	−0.05	−0.17	−0.37	−0.14	1.00	−0.29	0.22
Temperature	0.04	−0.78	−0.58	−0.5	0.02	−0.29	1.00	0.38
pH	0.08	−0.46	−0.44	−0.54	−0.09	0.22	0.38	1.00

be involved in several community outbreaks (Mellou et al. 2013). Moreover, since in a limited number of samples human pathogenic viruses (HAV) were detected, the risk of more severe waterborne viral infections should also be considered. These findings show that the potential of using human adenoviruses as indicators of the virological quality of water is emerging as has been previously suggested (Pina et al.1998; Bofill-Masv et al. 2006, 2010; Wyn-Jones et al. 2011; Rodríguez-Lázaro et al. 2012). In the context of a Europe-wide surveillance study which was carried out to determine the frequency of occurrence of hAdVs and NoVs in recreational waters, 553 out of 1410 samples (39.2%) were positive for one or more of the target viruses (Wyn-Jones et al. 2011). Interestingly, in the same study, comparisons with Fecal Indicator Organisms (FIO) thresholds defined in the European Directive bathing water standards (2006/7/EC) suggested that over 50% of samples that were relatively clean in terms of FIO concentrations and which exhibit "good" water quality, with a low associated illness risk, could be positive for hAdVs and NoVs (Wyn-Jones et al. 2011). It is extremely important to evaluate the public health risks associated with environmental virus hazards. Quantitative Viral Risk Assessment (QVRA) is a valuable statistical tool which can be used to estimate the probability of a viral infection, and involves the steps of virus hazard identification, exposure assessment, hazard characterization and risk characterization (Rodríguez-Lázaro et al. 2012).

Previous studies, based on benthic macrofauna as well as on spatiotemporal diversity of 16S rRNA, suggested that either the intrusion of seawater from the Ionian Sea and the circulation pattern diminishes the human impact in Igoumenitsa gulf or that there is no permanent disturbance (Karayanni et al. 2015; Meziti et al. 2015). Through the study of viruses, we 'witnessed' anthropogenic disturbance, since hAdVs were occasionally detected at all marine sampling sites and HAV was detected in sampling site 1 (S1, old port). These findings were probably related to the functioning of the wastewater treatment plant which cannot guarantee the absence of viral pathogens in the effluents or illegal flows of untreated urban sewage. Interestingly, no freshwater or sediment influences were observed through the study of 16S rRNA diversity using next generation sequencing (Meziti et al. 2015). However, it should be considered that the gulf of Igoumenitsa hosts important activities of shipping, aquaculture, and tourism which were not monitored but which probably affected the virological quality status of the area.

Kalamas river can be defined as an effluent-dominated stream since it is greatly affected by urban, agricultural, and industrial effluents (Lambropoulou et al. 2001; 2002; Kagalou et al. 2012; Nannou et al. 2015; Meziti et al. 2016). It is connected with Lake Pamvotis (Ioannina) through a narrow channel, named Lapsista, receiving the natural outflow of the lake as well as treated urban effluents from the city of Ioannina (150,000 inhabitants) and untreated sewages from medium and small size settlements along the river. In the present study, the highest counts for *E. coli* and enterococci did not exceed the set limits of "sufficient" quality, according to Council Directive 2006/7/EC. During a 12-month period (June 2007–May 2008), the prevalence and susceptibility of *Salmonella* serovars and their relation to specific pathogenic and indicator bacteria in Kalamas river waters were investigated, and 28 serovars of *Salmonella* spp. were identified (Economou et al. 2013). Although the drainage area of the river is characterized by high agricultural and livestock activity including cattle and poultry farms, no *Campylobacter* spp., *E. coli O157:H7, Cryptosporidium* and *Giardia* were isolated from the river water samples (Economou et al. 2013). In the present study, neither porcine adenoviruses (pAdVs) nor bovine polyomaviruses (bPyVs) were detected in any of the analyzed samples. In accordance to previous studies (Economou et al. 2013; Lekka et al. 2004), human interference was identified mostly at the lower part of the river, although most of the samples were found positive for hAdVs, thus indicating human fecal contamination. The highest hAdVs concentrations were found at K2 and K3 sampling sites, where human NoVGII were also detected, while human NoVGI were found only at K2 sampling site. Virological prevalence data in our study could be attributed either to non-point fecal inputs downstream Lapsista ditch or to Lapsista contributions. A metagenomic analysis of Kalamas water samples, conducted by Meziti et al. (2016), highlighted the role of allochthonous inputs, and revealed also the influence of Lapsista ditch effluents downstream to the estuaries (Meziti et al. 2016). Interestingly, in February, when the dam was open, all samples were negative for the different viruses tested during the study. This is probably related to the high water flow (261.28 m^3/s^1, mean annual flow: 54 m^3/s) which enhanced dilution (Ma et al. 2015) improving thus virological quality. Assessment of the ecological quality of Kalamas river based on macroinvertebrate data indicated poorer status during the low flow season compared to the high flow season (Lekka et al. 2004). High virus concentration before the dam, at K2 sampling site, compared to the other two sampling sites, K1 and K3, may be also related to its functioning parameters (accumulated water, retention time, water flow). It is known that dams may alter the species composition and population dynamics of microbial communities and that microbial abundance directly relates to the prevalence of infectious waterborne disease. Thus, any effect of dams on microbial community composition has immediate implications for human health. Previous studies have shown the effect of dams on distribution of bacterial pathogens due to the accumulation of water upstream and in relation to solar ultraviolet radiation conditions (Gorbatkin 2006; Mulamattathil et al. 2014).

In the study of Wyn-Jones et al. (2011), out of 1410 samples analyzed, 553 (39.2%) were positive for one or more of the target viruses, with hAdVs detected in 36.4% of samples. HAdVs were more prevalent compared to NoVs (9.4%), with 3.5% GI and 6.2% GII, some samples being positive for both GI and GII. (Wyn-Jones et al. 2011) In the canals and in nearshore water sites of the Florida Keys, 79% of sample sites were positive for enteroviruses; 63% were positive for HAV, and 11% were positive for Norwalk viruses, as reviewed by Griffin et al. (2003). A total of 95% of sites were positive for at least one of the viral groups overall. In Southern California, 33% (4 of 12) of marine samples were positive for adenoviruses. As reviewed by Griffin et al. (2003), these marine sites were located outside of river discharge points, and the authors noted that as in the Florida studies, bacteria indicators did not correlate with the presence of viruses. The applicability of human and animal adenoviruses and polyomaviruses in widely diverse river catchments in Europe and South America, to define sources of fecal contamination, was described by Rusiñol et al. (2014).

Although the number of analyzed water samples was overall low, the study revealed fecal contamination of human origin, in both Kalamas river and Igoumenitsa gulf. Human adenoviruses are reliable indicators of fecal contamination of human origin, and their detection implies a possible detection of other pathogenic viruses, such as HAV, NoVs. As the infectious dose of these viral pathogens is very low, their detection at specific sampling sites during the present study underlines waterborne infection risks. Moreover, these pathogens could be also present to the sampling sites which were found positive only for index viruses, further supporting the concerns for waterborne infections.

Overall, the present study contributed to the collection of useful data for the biomonitoring of the region, and the assessment of human impact. It also provided valuable information for the evaluation of the risk of waterborne viral infections and the protection of public health. It was the first virological study in the area and one of the few in Greece. The combination of data on virological quality with data concerning benthic fauna, water column organisms, abundance and genetic diversity of prokaryotes in water column (Meziti et al. 2015), and abiotic characteristics of water column, is expected to support (a) an integrated public health risk assessment, and (b) a management action plan design for the management of waterborne infection in the study area. Interestingly, the study could be used as a pilot project for other marine ecosystems in Greece and elsewhere.

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