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BRIEF COMMUNICATION

Assessment of the Incidence of Human Adenovirus in Surface Waters of Southwest Greece: Vouraikos River as a Case Study

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Abstract
The purpose of this study is to assess the overall impact of different anthropogenic activities in the Vouraikos River basin (southwestern Greece, Natura 2000 area). Virological quality of river water samples was investigated. Positive samples for human adenoviruses were found occasionally, while porcine adenoviruses and bovine polyoma viruses were not detected. It is the first time that virological data are collected in the study area.

Keywords Human adenovirus · Assessment · Vouraikos River · Public health · Karst terrain

Introduction

Rivers are dynamic and diverse ecosystems, serving as recipients of natural and anthropogenic inputs as they move downstream. These influences should be identified and qualified in order to prevent and reduce pollution, promote sustainable water use, protect the aquatic environment, improve the status of aquatic ecosystems, and mitigate the effects of floods and droughts, as it is designated by the Water Framework Directive (EC, 2000).

In the present study, we focused on Vouraikos River, which is located in the region of Peloponnese, southwestern Greece, and belongs to a Natura 2000 area. The most important aquifers of the area are developed in the limestone formations of two geotectonic zones, Olonos-Pindos and Gavrovo-Tripolis units. The Gavrovo-Tropolis limestones present an extensive network of conduits due to karstification, while the Olonos-Pindos limestones are distinguished by fissures and fractures where aquifers develop. The human activities in the area are mainly industrial and livestock activities, such as milk industries, stables, and fish farms that are potential sources of pollution. Nowadays, the karst aquifers are, both in quality and quantity terms, the most important groundwater resources in the Mediterranean countries, since most of their largest coastal cities are supplied with drinking water by such aquifers (Bakalowicz et al. 2003). In such areas, groundwater moves through conduits and fractures that are enlarged by dissolution processes. Moreover, the unsaturated zone of the aquifers does not have important self-cleaning ability, whereas any impairment of contamination is mainly due to dilution and hydrodynamic dispersion of pollutants. This along with the fact that there are extensive exokarstic features e.g., caves and in some cases interconnection between the surface- and ground-waters of the area makes the assessment of river water quality even more urgent (Civita 2008; Katsanou et al. 2017; Katsanou 2018).

An ecological risk assessment of cheese whey effluents has been previously performed in Vouraikos River (Karadima et al. 2010). Extensive studies of another Greek river, Kalamas, (located in NW Greece), verified 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 (Kagalou et al. 2012). In our recent study, of the virological quality of surface running water samples collected from Kalamas River, most of the samples were found positive for human adenoviruses (hAdVs), while human noroviruses GI and GII were also detected (Kokkinos et al. 2018).

The presence of enteric viruses in sewage and hence in environmental surface waters reflects the infectious status of the population and constitutes a public health risk. While fecal contamination of the water environment

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is currently being monitored only with bacterial indicators, it is nowadays well known that bacterial and viral contaminations are not necessarily correlated (Pina et al. 1998, Albinana-Gimenez et al. 2006; Bofill-Mas et al. 2006; Rusiñol et al. 2014; Bouwknegt et al. 2015).

Adenoviruses (AdVs) are the most prevalent of enteric viruses in water environments worldwide, they 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).

In the present study, we aimed at the assessment of the overall impact of different anthropogenic activities in the Vouraikos River basin by investigating the virological quality of river water samples, along with the collection of other microbiological data on classic bacterial indicators. Human adenoviruses were used as index viruses for the assessment of Vouraikos River virological quality, along with porcine adenoviruses and bovine polyoma viruses which are useful molecular source tracking (MST) tools for tracing the source of fecal viral contamination of human or animal origin (Rusiñol et al. 2014; Hundesa et al. 2009, 2010, Bofill-Mas et al. 2006; Pina et al. 1998).

Materials and Methods

Study Area

The study area is located in the region of Peloponnese, southwestern Greece, between 21°58′ and 22°18′ longitude and 37°45′ and 38°12′ latitude. The overall extent of the National Park is approximately 550 km^2. It displays a karst area and is mainly structured by the formations of Olonos-Pindos and Gavrovo-Tripolis units. A geological map of the study area is presented in Fig. 1a, while the location of potential sources of pollution in relation to rivers of the study area is shown in Fig. 1b.

The Vouraikos River has a length of 37 km, rises from Aroania Mountain and flows into the Gulf of Corinth. A Natura 2000 area, Vouraikos Gorge, about 20 km long, is located downstream, characterized by dense vegetation. The protection of its biodiversity is obligatory according to the European Directive 92/43/EEC.

Sampling Campaigns

Two sampling campaigns were performed, one during the dry period, in April 2013 (15 samples), and one during the wet season, in September 2013 (16 samples). Samplings were performed at "sensitive" sites located downstream of identified potential point sources of pollution (like livestock, fish farms,

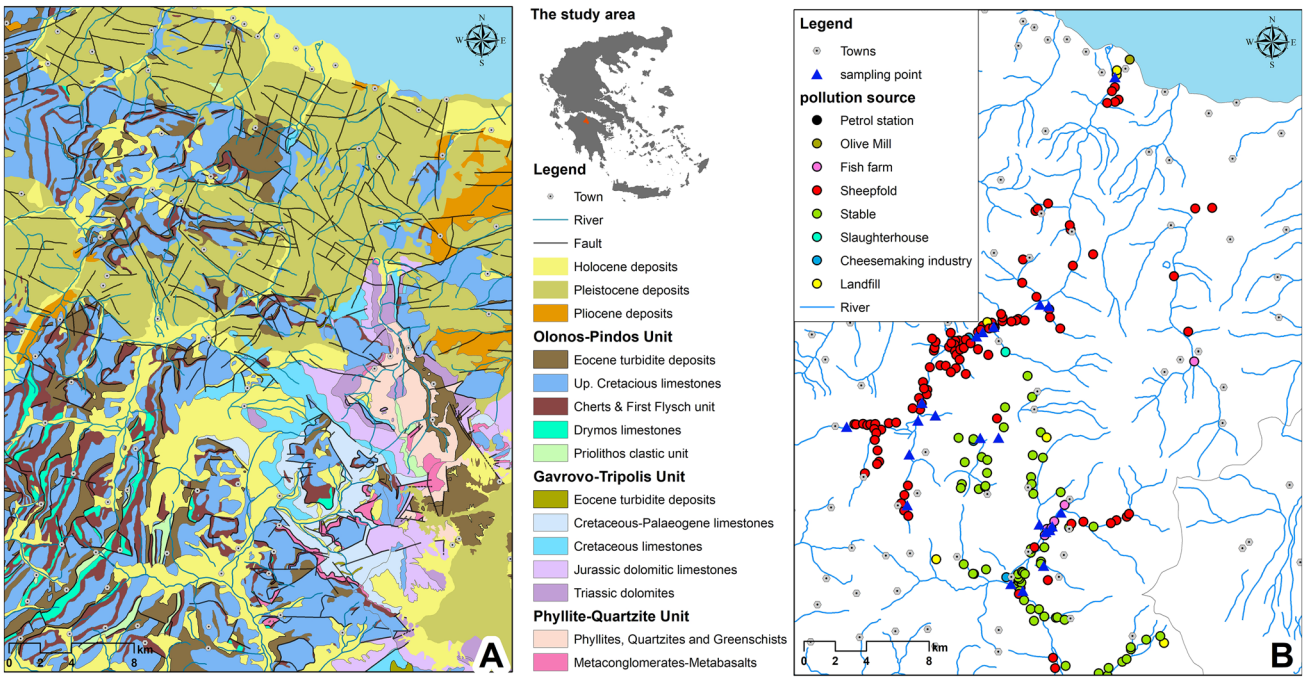

Fig. 1 **a** Geological map of the study area. **b** Location of potential sources of pollution in relation to rivers

Table 1 Description of sampling sites

No	Sampling site	Sampling location
1	Vouraikos	Bovine stable
2	Vouraikos	Cheese association
3	Vouraikos	Cheese factory
4	Vouraikos	Kalavrita pipeline
5	Vouraikos	Downstream of Kalavrita town
6	Kleitorios	Kleitoria output
7	La.donas	Cheese factory
8	Kleitorios	Biological treatment plant
9	Kleitorios	Downstream of cheese factory
10	Chandakas	Downstream of fish farming plant
11	Planitero stream	Downstream of fish farming plant
12	Planitero stream	Downstream of fish farming plant
13	Planitero stream	Downstream of fish farming plant
14	Vouraikos	Output of Vouraikos
15	Louson stream	Downstream of cheese factory
16[a]	Aroanios—Ladonas	River junction

[a]An additional sample (No16) was collected during the second sampling campaign of September 2013

cheese factories, and large villages) (Table 1). Total month rainfall ranged from 2 mm (dry season) to 31 mm (wet season).

Microbiological Analyses

Bacterial Indicators

Analysis for the classic bacterial indicators of river 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

Sample concentration, viral extraction, and biomolecular analysis were performed as described previously (Kokkinos et al. 2018). The analytical methods incorporated a sample process control (murine norovirus, MuNoV) and an internal amplification control. The nucleic acid extract was assayed neat, and in 10^{-1} dilution, and two replicate assays were performed for each concentration. (Kokkinos et al. 2015a, and references therein). The analysis method is accredited by ISO 17025 at the Environmental Microbiology Unit (EMU).

Statistical Analysis

Statistical analysis was performed by IBM SPSS v.24 statistical software (IBM SPSS Inc., USA). The statistical significance level was set at alpha = 0.05.

Results and Discussion

In total, 11 out of 31 samples (35.5%) were found positive for hAdVs. In detail, 6 out of 16 samples (40%) were found positive during the dry sampling period campaign, while 5 out of 16 samples (31.3%) were positive during the wet period campaign. Virological data per sampling site have been summarized in Table 2. Positive samples, for hAdVs (10 out of 11, 91%), were collected from identified "sensitive sites" located downstream of identified potential point sources of pollution, like livestock, fish farms, cheese factories, and large villages. These findings indicate the existence of fecal contamination of human origin. Enteric viruses in water may originate from run-off of animal manure, from discharges of raw or treated sewage, or directly from humans or animals (Rodríguez-Lázaro et al. 2012). In agreement with previous findings, hAdVs were all-season contaminants of the river (Sibanda and Okoh, 2012). In the present study, the number of samples tested for viral contamination was relatively small, and the presented results should be interpreted as indicative of human fecal contamination. Moreover, the monitoring, as applied in the current study, is more likely to detect structural contamination events rather than episodic contamination events. It should be noted that, sampling points that tested negative throughout the monitoring might be important for episodic viral contamination nevertheless. The negative results for the pAdVs and bPyVs index viral targets may be attributed to the relatively low number of analyzed samples, and the main prevalence of sheep and goat housing sites, instead of bovine and porcine.

The concentrations (cfu/100 ml) of *E. coli*, enterococci, and total coliforms, in Vouraikos River water samples, have been summarized in Table 2. Samples were found positive for *E. coli* at 90.3% (28/31), and for enterococci at 96.8% (30/31). Highest counts for *E. coli* and enterococci in river water samples exceeded the "sufficient" limits of 900 cfu/100 ml 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). The highest concentrations of *E. coli* (3500 cfu/100 ml), and enterococci (2400 cfu/100 ml), were found during the dry period campaign at the sampling site No 2. (Vouraikos, Cheese association). 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, though the study sample size was too low to detect a meaningful effect (Rodríguez-Lázaro et al. 2012; Kokkinos et al. 2015a, b, 2018).

The exploitation, and in many cases the overexploitation of karst aquifers, the absence of the unsaturated zone,

Table 2 Concentration (cfu/100 ml) of *E. coli*, enterococci, total coliforms, prevalence data of human adenoviruses (hAdVs), and BOD values of water samples, for the sampling campaigns of April and September 2013

No	BOD (mg L^{-1})	Coliform (cfu/100 ml)	*E. coli* (cfu/100 ml)	Enterococci (cfu/100 ml)	Human adenovirus (hAdVs)
1	2.5	150	80	15	Neg (−)
2	2.5	5800	3500	2400	Neg (−)
3	2.5	8300	3300	2000	Neg (−)
4	2.5	4000	400	1400	Neg (−)
5	2.5	870	170	80	Pos (+)
6	2.5	113	45	25	Pos (+)
7	2.5	73	< LOD	< LOD	Pos (+)
8	11	970	390	31	Neg (−)
9	2.5	270	30	32	Neg (−)
10	2.5	140	< LOD	1	Neg (−)
11	2.5	45	5	1	Neg (−)
12	2.5	150	30	37	Pos (+)
13	2.5	37	7	1	Pos (+)
14	2.5	140	60	9	Pos (+)
15	2.5	99	< LOD	7	Neg (−)
Second sampling campaign (September 2013)					
1	3	350	70	80	Neg (−)
2	3	5000	3000	160	Neg (−)
3	17	3400	2000	70	Neg (−)
4	10	3800	800	120	Neg (−)
5	3	110	70	32	Neg (−)
6	3	460	140	230	Pos (+)
7	3	86	9	46	Pos (+)
8	3	8200	2500	920	Pos (+)
10	3	19	114	12	Neg (−)
11	3	160	110	64	Neg (−)
12	3	110	90	75	Pos (+)
13	3	8	78	26	Neg (−)
14	3	1100	100	60	Neg (−)
15	7	3400	300	170	Neg (−)
16	3	7600	800	1100	Pos (+)

coupled with the burden of their quality due to human activities led many researchers, to place particular emphasis on the study of the vulnerability of karst systems to pollution (Panagopoulos and Lambrakis 2006; Civita 2008; Katsanou et al. 2017; Katsanou 2018). The present study focuses on a karst area.

The presence of the index viruses in the samples tested confirms that (a) viral contamination routes exist from source to monitoring points which pathogenic viruses could follow; and (b) the use of these index viruses is a valuable MST (Molecular Source Tracking) tool for tracing the source of fecal viral contamination (Kokkinos et al. 2011). Overall, the present study contributed to the collection of useful data for the biomonitoring of this karst region, and the assessment of the overall impact of anthropogenic activities.

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