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SEASONAL INFLUENCE ON THE PRESENCE AND PERSISTENCE OF ENTERIC VIRUSES IN WATER AND SEDIMENTS

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Por

Elmahdy Mohamed Elmahdy Ibrahim

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I dedicate this work to
my dear parents, Mohamed and Merfat,
and my wife Riham and kids Marwan and Rawan,
Who support me always.

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Education is the passport to the future, for tomorrow belongs to those who prepare for it today.

Malcolm X

RESUMO

A ocorrência de vírus entéricos no ambiente (água, solo) e em alimentos é bem documentada e tem sido associada a diversos surtos de gastroenterite de origem não bacteriana. Diversos patógenos virais foram identificados nestes surtos, incluindo norovírus (NV), vírus das hepatites A e E (HAV e HEV, respectivamente), astrovírus (AstV), poliovírus (PV), poliomavírus (PyV), adenovírus (AdV) e rotavírus (RV). Padrões de qualidade da água, tanto para consumo quanto para recreação, ainda se referem aos coliformes totais e termotolerantes e esse fato tem sido cada vez mais questionado pela comunidade científica e autoridades sanitárias no mundo todo.

Este estudo teve como objetivo avaliar o nível de contaminação por vírus em amostras de água e sedimento coletadas em duas regiões da ilha de Florianópolis em Santa Catarina, sul do Brasil: Rio Sangradouro (amostras de água de superfície e sedimento) e Lagoa do Peri (água de superfície, distintas profundidades de coluna de água- 0.9, 5.5 e 8.0 metros e sedimentos). Os vírus avaliados foram: adenovírus humano (HAdV), vírus da hepatite A (HAV), rotavírus grupo A (RVA), e colifagos somáticos. Para mimetizar a influência da radiação solar na estabilidade dos vírus montou-se um microcosmo em condições naturais de claro/escuro (dia e noite) e totalmente no escuro ambos em escala laboratorial e utilizamos AdV recombinante (rAdV) e norovirus murino (MNV-1) como modelos de vírus de genoma DNA e RNA (Capítulo III da tese). Os ensaios envolveram técnicas moleculares de PCR em tempo real (HAdV, HAV, RVA) para quantificação de genomas e técnicas de infecção celular *in vitro* (HAdV, rHAdV e MNV-1), além de técnicas microbiológicas clássicas (colifagos) para estudos de infecciosidade viral . **Capítulo I:** No ano de 2013 foram coletadas e analisadas um total de 96 amostras de água e sedimento. Para as amostras de água, HAdV foi detectado em 70,8% das amostras de verão, sendo que 82,4% dessas continham vírus infecciosos; a frequência do HAdV no inverno foi de 62,5% e nenhum deles continham vírus infecciosos. Para as amostras de sedimento, a frequência de HAdV foi de 37,5% nas amostras de verão, com 66,7% desses contendo HAdV infeccioso; a frequência HAdV no inverno foi de 37,5%, e nenhuma amostra continha vírus infecciosos. RVA foram detectados em 20,8 e 45,8% das amostras de águas superficiais coletadas no verão e inverno, respectivamente, e em 8,3 e 12,5% das amostras de sedimentos coletados no verão e inverno, respectivamente. Genomas de HAV foram detectados apenas em águas de superfície, com 54,8 e 12,5% de positividade em amostras de verão e inverno, respectivamente. **Capítulo II:** No ano de 2014 foi avaliada a

distribuição espacial de vírus em diferentes profundidades da coluna de água bem como sedimento. Um total de 84 amostras de água e 48 amostras de sedimentos foi analisado. Foi observado que, 64% e 48% das amostras de água e sedimento foram positivas para HAdV, respectivamente, sendo que 76% e 83% dos amostras respectivamente, continham vírus infecciosos. RVA estava presente em 33% e 18,75% das amostras de água e sedimento respectivamente e 25% das amostras de água foram positivas para HAV. Colifagos somáticos puderam ser detectados em 42% e 18,75% das amostras de água e sedimento, respectivamente. Os dados apontaram para uma variação de prevalência vírus de acordo com as diferentes profundidades da coluna de água. **Capítulo III:** As taxas de decaimento (*K*) e T_{90} para rHAdV e MNV-1 na água e sedimentos foram calculadas usando um microcosmo em escala de laboratório. Foram calculadas as unidades infecciosas de rHAdV e MNV-1 por microscopia de fluorescência e ensaio de placa de lise, respectivamente ao longo de um período de 85 dias. O curso do decaimento natural de rHAdV e MNV-1 foi similar sob luz solar natural nos microcosmos onde o T_{90} para rHAdV foi de 7,7 e 7 dias em água e sedimentos, respectivamente. O T_{90} para MNV-1 foi de 6,7 e 6,4 dias na água e sedimento, respectivamente. Nos microcosmos mantidos completamente no escuro observou-se uma estabilidade maior tanto para o rHAdV em água (T_{90} = 20,9 dias), quanto em sedimentos (T_{90} = 22 dias) sendo que para MNV-1 a estabilidade foi T_{90} = 12 dias e T_{90} = 18 dias em água e em sedimento, respectivamente. Estes resultados demonstraram a presença dos vírus entéricos potenciais causadores de doenças no principal manancial de água doce da Ilha de Santa Catarina e no Rio que corta muitos bairros dessa região e desemboca na Praia do Matadeiro, muito frequentada por banhistas e surfistas durante todo o ano e também a adsorção e estabilidade dos vírus aos sedimentos que são ressuspendidos pela ação dos ventos e chuvas, voltando às colunas de água. Esperemos que, no futuro, essas áreas sejam mais protegidas pelas autoridades e que a população se conscientize de seu papel na proteção dos recursos hídricos, fundamentais à manutenção da vida no planeta terra.

Palavras-chave: vírus entéricos, microcosmo, sedimento, colifagos somáticos, estabilidade viral, água de superfície, profundidade da coluna de água.

ABSTRACT

The presence of enteric viruses in the environment (water, soil) and in foods is well documented and has been associated with several gastroenteritis outbreaks of non-bacterial origin. Several viral pathogens were identified in these outbreaks, including Norovirus (NV), hepatitis A and E viruses (HAV and HEV respectively), astrovirus (AstV), poliovirus (PV), polyomavirus (PyV), adenovirus (AdV) and rotavirus (RV). Water quality standards, both for consumption and for recreation, still refer to the total and fecal coliforms and this fact has been increasingly questioned by the scientific community and health authorities worldwide.

This study aimed to assess the level of viral contamination in water and sediment samples collected from two regions: Rio Sangradouro (surface water and sediment samples) and Peri Lagoon, representing the main freshwater reservoir of the island of Florianopolis in Santa Catarina, southern Brazil, (surface water, different depths of water-column: 0.9, 5.5 and 8.0 meters and sediment). Evaluated virus were: human adenovirus (HAdV), hepatitis A virus (HAV), rotavirus group A (RVA), and somatic coliphages (Chapters I and II of this thesis). To mimic the influence of solar radiation on the stability of the virus it was set up a microcosm in natural conditions of light/dark (day and night) and totally in the dark both at laboratory scale and used recombinant AdV (rAdV) and murine norovirus (MNV -1) as models for DNA and RNA genome viruses (Chapter III of this thesis). The assays involved molecular PCR techniques in real time (HAdV, HAV, RVA) for quantification of genomes and cell culture techniques *in vitro* (HAdV, rHAdV and MNV-1) in addition to classical microbiological techniques (coliphages) for studies of viral infectivity**. Chapter I:** We collected a total of 96 samples of water and sediment during the summer and winter of 2013. For water samples, HAdV was detected in 70.8% of the summer samples, 82.4% of those with infectious virus; the incidence of HAdV in winter was 62.5% and none were infectious. For sediment samples, the incidence of HAdV was 37.5% in the summer samples, with 66.7% containing infectious HAdV; the incidence HAdV in winter was 37.5% and none were infectious. Genomes of RVA were detected in 20.8 and 45.8% of surface water samples collected in summer and winter, respectively, and 8.3 and 12.5% of the samples of sediment collected in summer and winter, respectively. Genomes of HAV were detected only in surface waters, with 54.8 and 12.5% positivity in summer and winter samples, respectively. **Chapter II:** The spatial distribution of the

evaluated viruses in different depths of the water column was evaluated. A total of 84 samples of water and 48 samples of sediment were analyzed. After analysis, 64% and 48% of water and sediment samples were positive for HAdV, respectively 76% and 83% respectively infectious. RVA was present in 33% and 18.75% of the samples of water and sediment respectively and 25% of water samples were positive for HAV. Somatic coliphages could be detected in 42% and 18.75% of water and sediment samples, respectively. The data indicated a variation in the virus prevalence according to the different depths of the water column. **Chapter III:** The decay rates (*K*) and T_{90} were calculated for rHAdV and MNV-1 in water and sediments using a microcosm in a laboratory scale. The infectivity was measured by infectious units of rHAdV and MNV-1 by fluorescence microscopy and plaque assay, respectively, over a period of 85 days. The course of natural decay of rHAdV and MNV-1 was similar under natural condition in microcosms where T_{90} to rAdV was 7.7 and 7 days in water and sediment, respectively. The T_{90} in NVM-1 was 6.7 and 6.4 days in the water and sediment respectively. In microcosms completely in the dark condition it was observed an increased stability for rHAdV in water (T_{90} = 20.9 days) and in sediment (T_{90} = 22 days) as well as for MNV-1 with T_{90} = 12 days and T_{90} = 18 days in water and sediment respectively. These results demonstrate the real presence of enteric viruses which cause diseases, in the main fresh water source of the island of Santa Catarina and the Sangradouro River that crosses many districts of this region and flow into Matadeiro Beach, which is used by swimmers and surfers throughout the year. Also we realize the adsorption and stability of the viruses to the sediments that are subsequently resuspended by wind and rain, returning to the water columns. Hopefully, in the future, these areas will be more protected by the authorities and the population will be aware of its role in the protection of water resources, essential to the maintenance of life on planet earth.

Key words: Enteric viruses, Microcosm, Sediment, Somatic coliphage, Viral stability, Surface water, Water column depth

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LIST OF ABBREVIATIONS AND ACRONYMS

A549 Epithelial cells from human lung carcinoma
ABES (Associação Brasileira das Empresas de Consultoria e Engenharia Ambiental), Brazilian Association of Consulting Firms and Environmental Engineering
AdV Adenovírus
APHA American Public Health Association
CASAN Catarinense Company for Water and Sanitation
CCL Candidate List of Contaminants
cDNA complementary DNA
CNPq National Counsel of Technological and Scientific Development"
CONAMA (Conselho Nacional do Meio Ambiente) National Environmental Council
CPE Cytopathic Effect
DMEM Dulbecco's modified Eagle medium
DNA Deoxyribonucleic acid
DNAse Desoxirribonuclease
DNTP Deoxyribonucleotides (dATP, dTTP, dGTP)
EDTA Ethylenediamine tetraacetic acid
EPA Environmental Protection Agency
FBS Fetal bovine Serum
FFU Focus Forming Unit
GC Genome copies
GFP Green Florescent Protein
HAdV Human adenovírus
HAV Hepatitis A virus
HCl Hydrochoric Acid
HEK human embryonic kidney
HEPES Hydroxyethyl Piperazineethanesulfonic Acid
IBGE Brazilian Institute of Geography and Statistics
ICC-et-RT-qPCR Cell Culture Integrated of Reverse transcriptase-Polymerase Chain Reaction in Real Time preceded by enzymatic treatment
ICC-RT-qPCR Cell Culture Integrated of Reverse transcriptase-Polymerase Chain Reaction in Real Time
K constant decay rate
LVA Laboratory of Applied Virology
MEM Minimum Essential medium with salts of Eagle's
MNV-1 Murine Norovirus 1
PBS Phosphate-Buffered Saline

PCR Polymerase Chain Reaction
PEG Polyethylene Glycol
PFU Plaque Forming Unit
pH Hydrogen potential
PMISB (Plano Municipal de Saneamento Básico) Municipal Plan for Sanitation
PNSB (Plano Nacional de Saneamento Básico) National Survey of Improved Sanitation
PSA Penicillin-Streptomycin-Amphotericin
QPCR Polymerase chain reaction in Real Time
RAW264.7 murine macrophage cell line
rHAdV recombinant Human Adenovirus
RNA Ribonucleic Acid
RT Reverse Transcriptase
RT-qPCR Reverse transcriptase-Polymerase Chain Reaction in Real Time
RVA rotavirus group A
SOMCPH Somatic Coliphage
$\mathbf{T_{90}}$ required time (days) to inactivate 90% of virus
UFSC Federal university Of Santa Catarina
USEPA United States Environmental Protection Agency
UV Ultraviolet radiation
VP Viral Protein
WHO World Health Organization

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LITERATURE REVIEW

LITERATURE REVIEW

1. PRESENTATION

This section will provide a brief literature review regarding the subject of this thesis.

1.1 WATER QUALITY IN BRAZIL

According to a report released on the occasion of the World Water Forum in Mexico, 2006, 57 million of the 6 billion people in the world lack safe drinking water. Brazil is one of the richest countries in the world in water resources, with about 13% of all fresh water in the planet. However today, the water distribution in Brazil meets more to agro-business interests than the people's needs. According to the Brazilian National Water Agency (AGÊNCIA NACIONAL DAS ÁGUAS-ANA) which is responsible for the National Water Resources Policy and creates the National System for Water Resources Management, the biggest water consumer in the country is the agro-business (72%), which has targeted production to export soybeans, beef and pork. For example, only in 2013, agro-business spent 200 trillion liters of water, equivalent to 200 full container systems. This is followed by industries with 22% while the household represent only 6% of water consumption in the country (AGÊNCIA NACIONAL DAS ÁGUAS-ANA, 2014)

According to ANA Brazilian Federal Law 9.433, from January 8, 1997, the term of water sources refers to surface water, underground water, flooding, or potentially usable waters for the public water supply, and the priority use is for human consumption and for animals drinking; Water resources management should always assure the multiple uses of water and the management of water resources should be decentralized and include the participation of public authorities, users and communities.

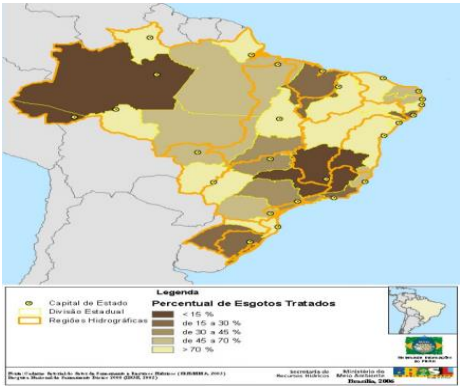
However, the water sources have been challenged with anthropogenic influences, and the pollution can be caused both by natural sources and humans, mainly through discharge of human, animal and industrial wastes. As this contaminated water can be reused in the field of agriculture uses such as irrigation, this can lead to degradation of food, soil quality and also will affect the quality of ground water (TUCCI et al., 2001). The non-collected and the collected but non-treated sewage are disposed in soils and water bodies, producing pollution. The sewage is the major cause of water pollution in Brazil.

For these reasons, sanitation and hygiene facilities are considered one of the most important concerns all over Brazil, to prevent or even reduce the risks of diseases related to release these contaminants into the aquatic environment (IBGE, 2008). Although, wastewater treatment processes are considered the most unsafe service in Brazil (IBGE, 2010).

According to the latest estimates of the WHO/UNICEF Joint Monitoring Program for Water Supply and Sanitation (JMP), 32% of the world's population – 2.4 billion people – lacked improved sanitation facilities, and 663 million people still use improper drinking water sources, in 2015 the vast majority living in India, China and Africa. As a result of inadequate access to safe water and sanitation services, 2.2 million deaths annual (mostly children under the age of 5) occur because of sanitation-related diseases and poor hygienic conditions (WHO; UNICEF, 2009 & 2015).

In Brazil, according to the National Survey of Improved Sanitation, known by the acronym PNSB there is a variable frequency survey according to the Brazilian Institute of Geography and Statistics (IBGE). Only 55.16% of the Brazilian municipalities have sewage collection network for services and, regarding Santa Catarina state, only 28.5% of the population have sewage treatment services (IBGE, 2008) as shown in Fig. 1.

Figure 1. Proportion of districts with wastewater treatment, according to Brazilian state and hydrographic basins. Brazil, 2000 (IBGE, 2008).



Source: Instituto Brasileiro de Geografia e Estatística

Currently the city of Florianópolis, Santa Catarina state, regarding the collection and treatment of sewage system, covers a total of 44% of the resident population (PLANO MUNICIPAL INTEGRADO DE SANEAMENTO BÁSICO – PMISB, 2008). This means that 56% of the resident populations are exhibited to the risk of water related diseases because of the possible route of aquatic system contamination by sewage disposal. In 1997 a real system of Wastewater Treatment Plant (WWTP) located in the south of the island, established by CASAN-Catarinense Company for Water and Sanitation (CASAN, 2011).

CASAN is a mixed capital company, established in 1970 by the State Law No. 4547 and incorporated on July 2, 1971, whose mission is to provide clean water collection and treatment of sewage. The company is present in 201 municipalities of Santa Catarina. Currently the services provided by CASAN cover almost the entire state of Santa Catarina, disposing of three water treatment plant (WTP) system called North, South and East Coast, Integrated in Florianópolis (PMISB, 2008). CASAN serves a population of 2.5 million people with treated water supply and 319,000 with sewage collection. Surface and ground water are used by CASAN as sources for water, treatment and distribution to the population. The water treatment process consists of: clarification (coagulation, flocculation, sedimentation and filtration), final treatment (disinfection, fluoridation and pH correction), reservation and distribution. In relation to sewage treatment consist of storage in Stabilization Ponds, Activated Sludge (oxidation, aeration) and Biological filters (CASAN 2011).

Quality and safety of water bodies in Brazil classified for human uses, according to Brazilian regulation, (THE NATIONAL ENVIRONMENT COUNCIL-CONAMA 2000) are evaluated according to bacteriological parameters which analyse only total and fecal coliforms (*E. coli*) as indicators for faecal contamination, which is required by Brazilian legislation (Portaria/MS 2.914/2011). However, there is no relationship between bacteria presence and other pathogens such as protozoa and virus detection because these last pathogens are more resistant to the most conventional treatment step either in water or wastewater treatment systems (FONG; LIPP, 2005). For these reasons, the Brazilian Association of Sanitary Engineering, known by the Portuguese-language acronym ABES (ASSOCIAÇÃO BRASILEIRA DE ENGENHARIA SANITÁRIA), suggested to include other microorganisms in the evaluation of water quality, including waterborne human viruses (ABES, 2010).

Contamination of coastal and estuarine waters by faecal pollution can lead to serious human health risks. According to the World Health Organization, about 1.8 million deaths from diarrhea occur each year; largely caused by unsafe water consumption (WHO, 2013). Coastal lagoons occupy around 13% of the coastal areas worldwide (KJERVE, 1994). These ecosystems are considered social, ecological and economically important due to their intensive use by humans and animals as water resources, sanitation tools and areas for swimming and recreation (SPAULDING 1994). Unfortunately, Brazilian coastal lagoons are subjected to several types of anthropogenic uses and influences due to population pressure (HENNEMANN; PETRUCIO 2011).

The lack of adequate sanitation in these ecosystems in coastal areas is a problem since in Brazil, one third of the population inhabits these regions (MINISTÉRIO DO MEIO AMBIENTE-MMA, 2007). As coastal communities grow, sewage can become a threat to local waterways: demand often exceeds available sewage treatment, and much of the sewage is dumped without being treated. Bathing in or ingesting sewage-contaminated water can cause infections and transmit diseases.

Sediments have their own hidden community of tiny inert and living components such as roots of living plants as well as populations of microorganisms and animals (VORONEY, 2007). Sediment covers more than 2-3 times of the earth's surface. There are two basic origins of the particles composing marine sediment 1) created *in situ* from the dissolved components, 2) carried by the water body through land or atmosphere.

The sediment is the result of soil erosion, and it was suggested to act as reservoir of pathogens from which can be released again into the water column as a result of natural or artificial phenomenon (ALM et al. 2003). Due to the high capabilities adsorption and accumulation phenomenon, their concentrations become several orders of magnitude greater than the corresponding overlaying water column.

Sediments are considered the only refuge for solid-associated viruses from the water column to settle in at the water's bottom, which may enhance the survival of these pathogens by reducing exposure to various stressors such as sunlight; it has been proven that viral abundance in the sediment exceeds that in the water column by an order of magnitude (DANOVARO et al., 1999; DANOVARO; SERRESI 2000; DANOVARO et al., 2002; DANOVARO; MIDDELBOE, 2010). Particles shed in fecal material in soil at low temperatures or in sediment

underwater will survive the longest and may be detectable for months or years (PERCIVAL et al., 2004). Recent studies suggested that sediment plays an important role in pathogen contamination by acting as a reservoir from which these pathogens can be re-released into the water column as a result of natural or artificial phenomena such as wind or swimming activity (SMITH et al., 1978; SCHAIBERGER et al., 1982; BOSCH, 1998; TOZE, 1999; SUBEKTI et al., 2002; ALM et al., 2003; SEARCY et al., 2006; SALVO; FABIANO, 2007).

1.2 Enteric viruses

The presence of pathogenic microorganisms in water generally results from pollution from human and animal faeces, from urban and rural wastewater (GONZALEZ et al., 1982). Whereas most waterborne pathogens have in common their origin in the faeces of sick individuals, a condition for assessing the microbiological quality of the water is the examination of indicators of faecal contamination (AMARAL et al., 2003). The most common microorganisms used as indicators are thermo tolerant coliforms, *Escherichia coli* and *enterococci* (ORTEGA et al., 2009). Unfortunately as we mentioned before, these bacteria cannot always be related to the presence of the virus, because they are also more persistent and stable than bacteria (PINA et al., 1998; LEES, 2000).

Enteric viruses enter into the environment by the wastes excreted by infected humans and animals. The aquatic ecosystem may also be contaminated as a result of sewage discharge or run-off from agriculture activities. (FONG; LIPP, 2005). Once introduced into coastal waters, they can easily absorb to solid particles, thereby protecting themselves from inactivating factors and may result in unpredictable resuspension to water column again due to natural or artificial sediment disruption (GERBA et al., 1977; SOBSEY et al., 1980).

The contamination of surface water by enteric viruses is one of the major public health concerns in relation to water resources used for drinking due to the health risks caused by these viruses. The destiny of these pathogens in the aquatic environment is controlled by sorption-desorption processes after they are being released to surface water (WONG et al., 2012 & 2013). Due to their size, these suspended particles seem to be the most important natural vehicle for these colloidal viruses in water column as a result of this phenomenon (GERBA et al., 1988; SCHWARTZBROD, 1995).

Recent studies suggested that sediments play an important role on pathogens spreading acting as reservoirs from which these pathogens

can be released again on water column as a result of natural or artificial phenomena such as wind or swimming activities (BOSCH, 1998; ALM, et al., 2003; SALVO; FABIANO, 2007; SEARCY et al., 2006).

Enteric viruses are major agents of gastroenteritis and hepatitis outbreaks in humans. Some of these viruses have been detected in water and sediment: human adenovirus (HAdV) which cause various diseases including gastroenteritis, upper and lower respiratory system infections, and conjunctivitis (WONG et al., 2013; JIANG et al., 2006; CALGUA et al., 2011, VERHEYEN et al., 2009, MIURA et al., 2009), rotaviruses (RVA) which is the major viral agent of severe diarrhea in children (GREEN; LEWIS, 1999; LE GUYADER et al., 1994) and hepatitis A virus which cause hepatitis (LE GUYADER et al., 1994; JOTHIKUMAR et al., 2000).

1.2.1 Adenovirus

Adenovirus constitutes a large group of DNA viruses that infect humans among other species (PEREIRA, 1963). The virus was first discovered in 1953 by Rowe and colleagues (ROWE et al., 1953) where they reported a viral agent causing degeneration of epithelial-like cells from tonsils and adenoids surgically removed from children. After Hilleman and co-workers isolated related agents from military personnel with respiratory illness in the United States (HILLEMAN et al., 1954). The viruses were first called adenoid degeneration (AD), adenoid-pharyngeal conjunctival (APC) and acute respiratory disease (ARD) agents, but in 1956 they were named adenoviruses (ENDERS et al., 1956). Adenoviruses were then associated with respiratory disease, acute hemorrhagic cystitis, gastroenteritis and epidemic kerato conjunctivitis (EKC). However, in 1962 it was demonstrated for the first time that adenovirus type 12 could induce malignant tumors in rodents (TRENTIN et al., 1962). Other serotypes (including Ad5) were found to be non-oncogenic.

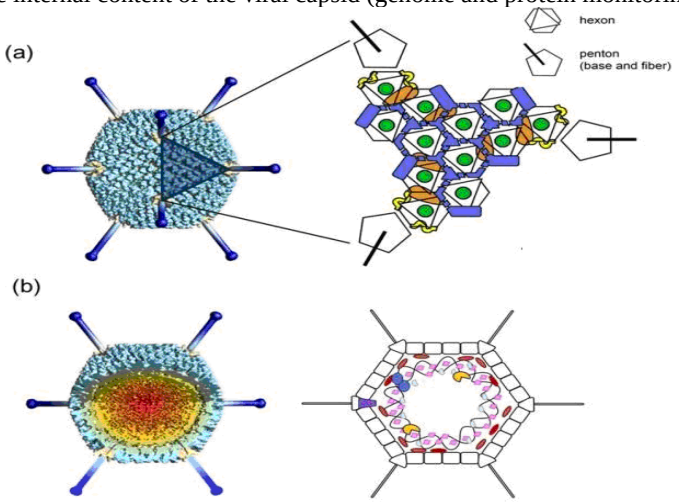
All Adenoviruses belongs to the family *Adenoviridae*, with icosahedral viral capsid which is divided into five genera: Mastadenoviruses infecting mammals, Aviadenovirus infecting birds, Siadenovirus infects birds and frogs and finally Atadenovirus infecting a broad range of hosts including avian, reptile and marsupial host (BENKO et al., 2000). A possible fifth genera infecting fishes has been proposed to be called Ichtadenovirus (BENKO et al., 2002) as shown in Fig. 2.

The genera are separated primarily on the basis of difference in immunological properties of the virions. There are fifty-six human

adenovirus serotypes and they have been distinguished on the basis of their resistance to neutralizing by antiserum to other known adenoviruses (DE JONG et al., 1999; CDC-National Center for Immunization and Respiratory Diseases, 2015).

Human adenoviruses cause infections of various severities. They are associated with different clinical syndromes which are caused by cytopathogenic effect on cells in the infected organs. Subgroup C which includes Ad type 5 is associated with respiratory illness (HORWITZ, 2007) and it is suggested that the disease is transmitted via aerosol. Other subgroups are associated with pharyngoconjunctival fever, epidemic keratoconjuntivitis, and gastroenteritis. These infections are suggested to be transmitted either via poorly chlorinated pools eye-to-hand-hand-to-eye contact (AZAR et al., 1996) and fecal-oral routes (WADELL, 1988; HORWITZ, 2007).

Figure 2: General structure of the adenovirus schematically represented: a) The shape of the icosahedral viral capsid with their hexons and pentons, b) and fimbriae internal content of the viral capsid (genome and protein monitoring).



Source: Adapted from MARTIN (2012).

HAdV causes many diseases such as gastroenteritis, urethritis, cervicitis, pharyngitis, acute respiratory diseases, conjunctivitis, and meningoencephalitis (FONG; LIPP, 2005). Of 56 serotypes of HAdV reported in the literature, replicate in the gastrointestinal tract and therefore can be excreted in high concentrations in feces (10^8-10^{13} particles / gram) (BANYAI et al., 2009). Since they are DNA viruses,

which promotes more resistance to environmental degradation, such as UV radiation, temperature, chlorine concentration and pH variation, including sewage treatment procedures (PINA et al., 1998, CARTER, 2005; LECHEVALLIER; AU, 2004; FONG; LIPP, 2005). Furthermore, HAdV have been described in drinking water (FONG et al., 2005; RIGOTTO et al., 2010; GARCIA et al., 2012), in recreational waters (MIAGOSTOVICH et al., 2008; SINCLAIR et al., 2009; FONGARO et al., 2012), associated with disease outbreaks in swimming pools (PAPAPETROPOLOU; VANTARAKIS, 1995; HARLEY et al., 2001), and in polluted waters (PINA et al., 1998; LAVERICK et al., 2004; LEE et al., 2004; MIAGOSTOVICH et al., 2008; WONG et al., 2009; RIGOTTO et al., 2010; MORESCO et al., 2012). Moreover, HAdV were found to be more prevalent than other enteric viruses in different aquatic environment (WONG et al., 2009; WYN-JONES et al., 2011; GARCIA et al., 2012; FONGARO et al., 2012; MORESCO et al., 2012). All together, these characteristics may provide a useful index of human faecal pollution and human viral contamination. Among other enteric viruses, adenovirus is considered a high priority emerging contaminant present in drinking water, candidate parameter contamination of the aquatic environment (USEPA, 2009).

The hexon is the largest capsid protein. The molecular mass is approximately 360kDa consisting of three identical subunits with 120kDa each (ATHAPPILLY et al., 1994). The hexon surface loops display the highest variability between adenovirus serotypes and contain most of the type specific epitopes (EBNER et al., 2005).

All Adenoviruses genomes are double stranded DNA of approximately 36Kb. After infection, the transcription of AdV has two mainly phases namely early (occur before replication) and late phases (occur after replication).

The entire adenovirus genome is a linear, double-stranded DNA of approximately 36Kb. There are approximately 2 phases of adenovirus transcription, named early and late phases, which occur before and after replication. The early-transcribed regions are E1, E2, E3 and E4. E1 include two gene products, E1A and E1B which are involved in the replication of this virus inside host cell. E2 acts as machinery for viral DNA replication and late genes transcriptions. E3 plays an important role in modulating the immune response of infected cells. E4 promotes virus DNA replication and shut-off synthesis of protein for the host cells (GRAHAM et al., 1977).

1.2.2 Rotaviruses

Rotavirus is the most common cause of viral diarrheal disease in both developed and developing countries worldwide, where they constitute a major cause of mortality among the young children. More than 500,000 children under five years of age still die from rotavirus infection each year (KAPIKIAN et al., 2001). Human rotaviruses were discovered in 1973 by Ruth Bishop and her colleagues by electron micrograph images (BISHOP et al., 1973). Rotavirus is a genus of double-stranded RNA virus within the family *Reoviridae*. Rotaviruses are icosahedral 65-70 nm particles, non-enveloped (resistant to lipid solvents), and the capsid contains all enzymes for mRNA production (KAPIKIAN et al., 2001).

There are eight species of this virus, referred to as A, B, C, D, E, F, G and H. Rotavirus A, the most common specie, causes more than 90% of rotavirus infections in humans. The rotavirus genome contains 11 segments of dsRNA, which have a size range of 0.6 to 3.3 kilobase pairs each (MATTION et al., 1994). These viruses are transmitted by faecal-oral route. In infected persons, after an incubation period of about two days, rotaviruses damage the cells that lining the small intestine and cause severe gastroenteritis. Symptoms often start with vomiting followed by four to eight days of profuse diarrhoea. Symptomatic infection rates are highest in children under two years of age and decrease in adults due to immunity that they acquired in their childhood. Transmission happens by contact with contaminated water, food, and surface or even by contact with contaminated persons. Rotavirus is very stable in the environment and may remain viable in the environment for weeks or months if not disinfected (WILDE et al., 1992; ABAD et al., 1994). Rotavirus shedding from the gastrointestinal tract prior to onset of diarrhea has been well documented in the literature (LUDIN et al., 2003).

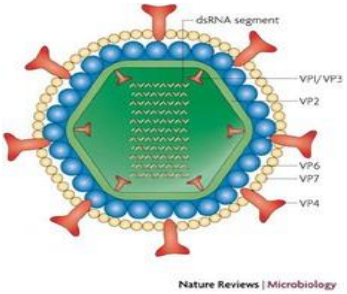
Zoonotic nature of rotavirus is reported in some cases worldwide. The segmented nature of the RV genome allows for gene reassortment upon co-infection of a single cell with 2 different RV strains and this may highlight the role of animal as a source of rotavirus infection to humans (NAKAGOMI; NAKAGOMI, 1993). Rotaviruses have been detected in raw or treated sewage (KARGAR et al., 2013).

There are six viral proteins (VPs) that form the virus particle (virion) as shown in Fig. 3. These structural proteins are called VP1, VP2, VP3, VP4, VP6 and VP7. In addition to the VPs, there are six nonstructural proteins (NSPs), which are only produced in cells infected by rotavirus. These are called NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6. The

glycoprotein VP7 defines the G serotypes and the protease-sensitive protein VP4 defines P serotypes (KIRKWOOD, 2010).

The virus is composed by three concentric shells that enclose 11 gene segments. The outermost shell contains two important proteins VP7, or G-protein, and VP4, or P-protein. VP7 and VP4 define the serotype of the virus and induce neutralizing antibody that is probably involved in immune protection. G1 [P8] genotype is the world's most prevalent, accounting for over 70% of RV-A infections in North America, Europe and Australia, but only 30% of RV-A infection in South America and Asia, and 23% in Africa (TATE et al., 2010).

Figure 3: Schematic representation of a rotavirus particle containing its major viral protein of the respective layers: VP2 (internal), VP4 (external) VP6 (middle) and VP7 (external) and ds RNA genetic material.



Source: Adapted from LI et al., (2009).

Animal and human RVs *in vitro* cultivation has been done by using of primary and transformed monkey kidney cells (HASEGAWA et al., 1982; SATO et al., 1981; WARD et al., 1991; WARD et al., 1984; WYATT et al., 1983; WYATT et al., 1980) Cultivation is achieved by pretreatment of virus with trypsin as a proteolytic activation (5 to 10 µg/mL). Many cell lines are used for isolation of rotavirus from the clinical samples such as simian MA104 rhesus monkey kidney cells and continuous human colon adenocarcinoma cell lines (CaCo-2 and HT-29) but at the same time there are many rotavirus out of group A proven to be quite fastidious and difficult to grow on cell lines (LI et al., 2009).

In the United States alone, 2.7 million cases of rotavirus gastroenteritis occurred yearly before initiation of the rotavirus vaccination. Rotavirus infections occur primarily during cool, dry seasons and the seasonal distribution of rotavirus infection has been extensively reported in many studies worldwide (KAPIKIAN et al.,

2001; MORESCO et al., 2012, RIGOTTO et al., 2010, SATO et al 2010; FONGARO et al., 2012).

Since 2006, two oral rotavirus vaccines have been licensed and available in North and South American, European and Eastern Mediterranean countries, where they have significantly reduced the burden of rotavirus-induced diarrhea.

In 2006, a live, oral, human-bovine reassortant rotavirus vaccine (RotaTeq®, produced by Merck and Company, Whitehouse Station, New Jersey) was recommended for routine vaccination of US infants. Three doses of this vaccine are recommended to be administered at ages 2, 4, and 6 month. RotaTeq® contains five reassortant rotaviruses developed from human and bovine parent rotavirus strains that express human outer capsid proteins of five common circulating strains (G1, G2, G3, G4, and P[8] (subgroup P1A))

In 2006, an attenuated monovalent G1,P[8] human rotavirus strain Rotarix® (produced by GlaxoSmithKline Biologicals, Rixensart, Belgium), and is recommended by the manufacturer to be orally administered in 2 doses to infants at ages 2 and 4 months. In Brazil, attenuated G1P [8] vaccine, was included in the Immunization Program since 2006 (FUMIAN et al., 2011).

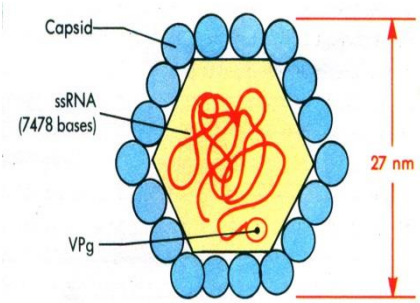
In 2014, Rotavac was licensed by the Drugs Controller General of India (DCGI). The vaccine originated in India from an attenuated (weakened) strain of rotavirus that was isolated from an Indian child (WHO, 2014).

1.2.3 Hepatitis A virus (HAV)

Feinstone and co-workers in 1973 (FEINSTONE et al., 1973) were identifies HAV by immune electron microscopy of infected persons feces. In 1979 the first trial for cultivating HAV in cell culture done by Provost and Hilleman (PROVOST; HILLEMAN, 1979). HAV is a member of *Picornaviridae* family, and is the only member and type species of the genus Hepatovirus. HAV is a nonenveloped virus, 27 to 32 nm in diameter with an icosahedral capsid (HOLLINGER; EMERSON, 2001) as shown in Fig. 4. Virion RNA is plus sense, single stranded and 7,478 nucleotides in length. An icosahedral capsid formed by several copies of three or four VP1 protein, VP2, VP3 and VP4, and the latter one, play an important role in the formation of the viral particle (HOLLINGER; EMERSON, 2001; PINTO et al., 2010). HAV has only one serotype and seven different genetic groups (four humans and three simian) (CRISTINA; COSTA-MATTIOLI, 2007).

Transmission of HAV may be occurring by ingestion of contaminated water and food or even by contaminated shellfish with the transmission via the fecal-oral. Following ingestion, HAV enters the bloodstream through the epithelium of the oropharynx or intestine and go directly to the liver, where it multiplies within hepatocytes. Virions are secreted into the bile and released in stool with large concentration after about 11 days from symptoms appearance.

Figure 4: Diagram of the picornavirus structure of the hepatitis A virus (HAV). The icosahedral capsid is made up of four viral polypeptides (VP1 to VP4). Inside the capsid is a single-strand positive sense RNA that has a genomic viral protein (VPg) on the 5'end.



Source: http://pathmicro.med.sc.edu/virol/hepatitis-virus.htm

HAV adapted in fetal rhesus monkey kidney cell line (FRhk 4), but HAV did not show any morphological changes on the infected cells. In 1978, MATHIESEN and co-worker (MATHIESEN et al., 1978) succeed to demonstrate HAV replication by direct immunofluorescence analysis. However, HAV is usually difficult to adapt and grow *in vitro* scale.

The clinical course may vary from asymptomatic, acute self-limiting hepatitis in patients with unapparent or subclinical hepatitis to life threatening acute liver failure (ALF) which occasionally develop fulminant hepatitis and die (HOLLINGER; EMERSON, 2001).

Poor sanitary conditions and regional socio-economic conditions are the main sources for outbreaks caused by HAV (VILLAR et al., 2006). HAV consider being endemic in Brazil and this is confirmed by many epidemiological data that show that 90% of Brazilian people have anti-HAV antibodies (CARRILHO et al., 2005).

Uncooked bivalves such as oysters and clams which harvested from water contaminated with sewage are responsible for many outbreaks

worldwide and this fact due to these shellfish able to filter large amount of water (contaminated by human sewage) per hour over a short period during their feeding where HAV can be concentrated at least 100-fold and persist for about 7 days (ENRIQUEZ et al., 1992; SÁNCHEZ et al., 2002; VILLAR et al., 2006).

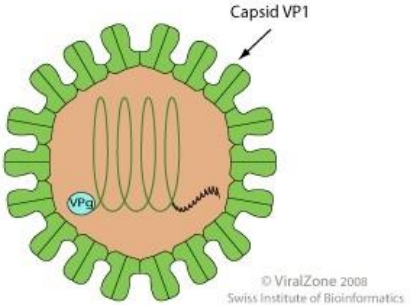
Many studies have shown the presence of HAV in aquatic environment including water treatment systems, lagoon and surface water since it has a chemical-physical characteristics that ensure their strength, it is not susceptible to degradation by detergents, relatively resistant to temperatures up to 60°C and inactivated partially when incubated for a period of 10 to 12 hours at this temperature and may still remain infectious for days or months in contaminated water (Cuthbert 2001; HOLLINGER; EMERSON, 2007).

There are two types of vaccines against HAV, the first one containing inactivated HAV (Havrix) and another containing a live attenuated virus (VAQTA) (IRVING et al., 2012). The vaccine protects against HAV in more than 95% of cases for longer than 25 years. The vaccine should be given in the muscle of the upper arm and be given in two doses for the best protection. An initial dose provides protection starting two to four weeks after vaccination; the second dose, given six to 12 months later, provides protection for over 20 years and the proven efficiency of vaccination, since there is only one serotype of HAV current worldwide (IRVING et al., 2012).

1.2.4 Norovirus (NVs)

Norovirus was identified in 1972 by Kapikian and colleagues (Kapikian et al., 1972), using immune electron microscopy. Noroviruses belong to *Caliciviridae* family, RNA single-stranded positive sense, non-enveloped with a diameter of approximately 27 nm. Contain three open reading frames (ORFs). The ORF1 encodes a large polyprotein which is cleaved at six other important proteins involved in viral replication, like the viral polymerase. ORF 2 encodes the structural protein VP1 and VP2 which are important for the ORF 3 protein. (ATMAR, 2010) as shown in Fig. 5.

Figure 5: schematic description of a viral particle, with emphasis on the HAV structural protein VP1



Source: Adapted from viral zone website (http://viralzone.expasy.org/viralzone/all_by_species/194.html, 2016)

Norovirus is a very contagious virus. It can be transmitted via dealing with infected persons, contaminated water or food, even by eating uncooked shellfish. Infection by norovirus can lead to serious gastroenteritis especially for young children and older adults. Worldwide, norovirus is the leading cause of gastroenteritis outbreaks. The most common norovirus strain (genogroup II genotype 4) is a major cause of outbreaks worldwide with an estimated 23 million cases in the United States of America each year, is transmitted by the fecal-oral route and by aerosol (SUFFREDINI et al., 2012; MESQUITA et al., 2011; TERIO et al., 2010; GENTRY; VINJE; LIPP, 2009).

Until this moment there is only one publication in the literature done by JONES et al., (2014) which describes the possible culture and replication of human norovirus *in vitro* with the presence of enteric bacteria that express antigens HBGA (histo-blood group antigen). However, the murine norovirus (NVM) was the only norovirus that showed *in vitro* replication capacity in cell culture (WOBUS; THACKRAY; HERBERT, 2006).

Murine norovirus (MNV-1) which was used in the fourth chapter of this work, has been widely used as a substitute and surrogate for human norovirus, it belongs to the same family and has great structural similarity and transmission route (CANNON et al., 2006). Murine norovirus has the ability to infect *in vitro* murine macrophage cell line RAW264.7, causing a clear cytopathic effect in about 36 h. The lysis assay plate is well standardized, providing information on viral viability

within 48 h (GONZALEZ-HERNANDEZ; CUNHA; WOBUS, 2012; PREDMORE; LI, 2011).

1.3 Coliphage as indicator for fecal pollution

Although enteric bacterial indicators have been used as acceptable tools to estimate the microbiological quality of environmental waters, many waterborne pathogens that cause illness and diseases in humans are enteric viruses. Microbial indicators such as total coliforms, fecal coliforms may be unreliable indicators for predicting enteric virus presence in contaminated water and the associated human health risks. Compared to bacterial indicators, enteric viruses have shown higher survival rates during wastewater and drinking water treatment and greater persistence in environmental waters (CONTRERAS-COLL et al., 2002; DURAN et al., 2002; HARWOOD et al., 2005; MOCE-LLIVINA et al., 2005; JOFRE et al., 1995).

Due to higher persistence and resistance of waterborne enteric viruses compared to bacterial indicators, water which is defined as safe based on bacterial indicator measurement may still cause disease or illness to consumers due to the enteric viruses still present (DORE et al., 2000; FORMIGA-CRUZ et al., 2003). Therefore, bacterial indicators alone do not provide a complete assessment of microbiological water quality. By using at least one or two viral indicator for prediction the presence of enteric viruses in the aquatic environment will help us to better assessment of water quality and provide more confidence on water safety.

There are several candidate viral indicators to predict virus contamination in environmental waters. Many studies advocated phages infected enteric bacteria as a potential viral indicator to estimate the microbial contamination in the water environment, also as indicators of water treatment process efficacy (HAVELAAR, 1993). Bacteriophages, specifically phages of Escherichia coli (Coliphages) are proposed candidate indicators of human enteric viruses in water. They are present in human and animal feces and some are small, icosahedral and non-enveloped viruses, making them structurally similar to enteric viruses. There are two main types of coliphages: somatic and the male-specific (F+).

The somatic coliphages are DNA viruses which are able to infect *E. coli* by attaching to specific sites on the outer cell membrane, such as lipopolysaccharide. The male-specific coliphages are single-stranded RNA and DNA viruses that infect the cell via the pili appendages present on the surface of male strains of the bacterium. Many studies in

the literature documented that male-specific coliphages play as viral indicators depending on their investigations (DORE et al., 2000; COLE et al., 2003; COLFORD et al., 2007; LOVE; SOBSEY, 2007). However, their infrequent presence in human feces, their relative scarcity and their rapid decay rates in high temperature waters (LOVE et al., 2007) limit their usefulness as indicator viruses. Somatic coliphages also have been proposed as fecal indicators of human enteric viruses in studies of sewage, source water for drinking water and marine waters (MOCE-LLIVINA et al., 2005; MUNIESA et al., 2007; JOFRE, 2008), and real-time monitoring of somatic coliphages as fecal indicators has been suggested by previous investigators (ARAUJO et al., 1997; SKRABER et al., 2004, GARCIA-ALJARO et al., 2008). So we include in this part of the work the detection and enumeration of somatic coliphage (PHI-X-174) as indicator of enteric viruses to try to make a possible correlation between them and the presence of the studied enteric viruses (HAdV, RVA, HAV).

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2. OBJECTIVES

2.1 GENERAL OBJECTIVE

To assess and quantify human enteric viruses and somatic coliphages in water and sediments samples in natural environment and in microcosm.

2.2 SPECIFIC OBJECTIVES

Chapter I

1- To standardize the method for virus concentration in sediment samples and to apply this optimized method in the field samples;

2- To quantify the genomic copies of human enteric viruses: rotavirus A (RVA), hepatitis A virus (HAV) and human adenovirus (HAdV) in surface water and sediments of the Sangradouro River and to correlate them with the physical-chemical parameters of the samples;

3- To evaluate the infectivity/viability and integrity of HAdV present in surface water and sediment samples.

Chapter II

1- To quantify the genomic copies and the spatial distribution of RVA, HAV and HAdV in the water column and sediment of Peri Lagoon in the spot used for water catchment for drinking purposes;

2- To correlate the presence of these viruses with the physical-chemical parameters of these samples;

3- To evaluate the infectivity/viability of HAdV and somatic coliphages present in water and sediment samples.

Chapter III

1- To evaluate the temporal stability of recombinant adenovirus (rAdV) and murine norovirus (MNV-1) in water and sediment samples using a microcosm in a laboratory scale *ex-situ*;

2- To evaluate the effect of natural U.V. sunlight on the stability of rAdV and MNV-1 in the laboratory scale microcosms.

CHAPTER I

DETECTION OF ENTERIC VIRUSES IN SURFACE WATER AND SEDIMENT SAMPLES OF SANGRADOURO RIVER

CHAPTER I

1. INTRODUCTION

This chapter will present and discuss the results regarding the enteric viruses assessment in water and sediment samples collected at Sangradouro River and beach of Peri Lagoon by real time PCR (qPCR) and also by cell culture techniques. These results were published in Journal of Water and Health.

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Enteric viruses in surface water and sediment samples from the catchment area of Peri Lagoon, Santa Catarina State, Brazil

E. M. Elmahdy, G. Fongaro, C. D. Schissi, M. M. Petrucio and C. R. M. Barardi

ABSTRACT

This paper aims to quantify human adenovirus (HAdV), rotavirus species A (RVA), and hepatitis A virus (HAV) in surface water and sediments and to determine the viability of HAdV in these samples. Water and sediment samples were collected, and HAdV, RVA, and HAV were quantified by real-time polymerase chain reaction (PCR); HAdV was also evaluated for infectivity by a plaque assay (PA). For the water samples, HAdV was detected in 70.8% of the summer collections, with 82.4% containing infectious HAdV; the HAdV incidence in winter was 62.5%. For the sediment samples, the incidence of HAdV was 37.5% in the summer collections, with 66.7% containing infectious HAdV; the HAdV incidence in winter was 37.5%. RVA was detected in 20.8 and 45.8% of surface water samples collected in summer and winter, respectively, and 8.3 and 12.5% of sediment samples collected in summer and winter, respectively. HAV was detected only in surface waters, with 54.8 and 12.5% positivity in summer and winter samples, respectively. This study demonstrated that enteric viruses are present in water and sediments and that the presence of infectious viruses should be investigated whenever possible for quantitative microbial risk assessment studies. Combined analyses of water and sediments are important for reliable public health risk analysis of recreational and lagoon waters.

Key words | coastal lagoon, enteric viruses, infectivity, quantitative PCR (qPCR), sediments, surface water

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2. MATERIAL AND METHODS

The general experimental design to perform the present work consisted in two stages: 1) monitoring enteric viruses (HAdV, RVA and HAV) during the summer and winter seasons of the year 2013 as well as the physical-chemical parameters in surface water and sediment samples of Sangradouro River and Peri Lagon; 2) evaluating HAdV infectivity for all positive samples detected by qPCR.

2.1 Description of the study area

2.1.1 Peri lagoon

The Peri Lagoon is a small coastal lagoon, located in southern Brazil, in Santa Catarina state, in the southeastern portion of Santa Catarina Island (27°44´S and 48°31´W), Florianópolis. The Peri Lagoon environment has suffered over several decades, a strong environmental degradation with an increasing population occupying their surroundings and landfills. Dumping of sewage is one of the most common forms of pollution coastal areas and the population living in the surrounding areas that are contaminated is affected, since many people use this environment as their source of livelihood and recreation. Furthermore, the contaminants released together with the sewage may accumulate in the sediment, including bacteria and viruses pathogens that cause risks to health (LEES, 2000; MUNIAN-MUJIKA et al., 2000) (Fig. 7).

The Peri lagoon has a surface area of 5.7 km^2, is surrounded by mountains, and currently constitutes the main source of drinking water for the island inhabitants because it has no direct seawater influence. Waters of the lagoon are drained into Sangradouro River in a unidirectional sense that extends towards the south of the island, crossing areas of urban occupation and emptying into the ocean to the east between the beaches of Armação and Matadeiro. The river waters are used to deposit illegal sewage discharge from neighborhood residences. The climate in the area is subtropical, with rainfall well distributed throughout the year (1.85 mm annual rainfall), although rainfall is more frequent during the summer months than during the winter (CECCA, 1996 and 1997). The lagoon was the first point of water collection as shown in Fig. 6 (a, b).

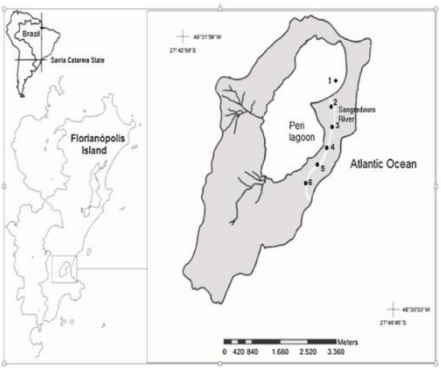
The second collection site, comprised of points 2 through 6, was in Sangradouro River. Points 2 and 3 had sediment that consisted of mainly sand and probably sewage, while points 4 and 5 had sediment that consisted of sand, silt, gravel, and probably sewage. Point 6, at the fourth sample collection area located at the end of Sangradouro River, was the collection point closest to the beach of Armação, and the

sediment of this point consisted of mainly sand and probably sewage as shown in Fig (7, 8, 9, 10, 11 and 12)

Figure 6 (a, b): Location of Peri Lagoon and the sampled sites along the Sangradouro River. Numbers indicate the sampling sites (coordinates for each site: 1:27◦43 31.91S/ 48◦30 35.91W; 2:27˚43.9631S, 048˚30.6081W; 3:27˚44.5401S, 048 ˚30.5961W; 4:27˚45.0431S, 048˚30.5421W; 5:27˚45.2241S, 048˚30.2561W; 6:27˚45.1171S, 048˚30.1431W

a)

b)



Source: adapted from HENNEMANN; PETRUCIO, (2011).

Figure 7: Site 1 of collection, Located on the beach of Peri Lagoon,

Figure 8: Site 2 of collection, Located at the beginning of Sangradouro River near to Peri Lagoon

Figure 9: Site 3 of collection, located about halfway through the Sangradouro River.

Figure 10: Site 4 of collection, Located in the district Armação.

Figure 11: Site 5 of collection near to many houses and population

Figure 12: Site 6 of collection, closest to beach Armação at the end of Sangradouro River.

2.2 Sampling

2.2.1 Water and sediment

A total of 48 surface water samples (about 10-15 cm in deep, 2 L each) were collected from the six points of collection during the summer and winter of 2013 year. Four collections were done for each season, with summer collections occurring during March/April 2013 and winter collections occurring during July/August 2013. Additionally, a total of 48 sediment samples (20 g was taken from each collection for analysis) were collected in parallel with the water samples at each collection point using ***Petersen*** grab samplers (provided by LAPAD- Laboratório de Biologia e Cultivo de Peixes de Água Doce) as shown in Fig. 13.

The samples were transported to the laboratory on ice in sterile containers and were immediately processed. A sum of 96 samples was collected from 6 sites during one year.

Figure 13: *Petersen* grab dredge used for sediment sample collection

Source: provided by LAPAD- Laboratório de Biologia e Cultivo de Peixes de Água Doce-UFSC)

2.3 Physical-chemical analysis

Water temperature (WT), conductivity (Cond), pH, salinity (Sal.) and dissolved oxygen (DO) were immediately measured *in situ* with specific probes (WTW-Multi350i) after collection, as is outlined in the standard methods for examination of water and wastewater (APHA, 1998).

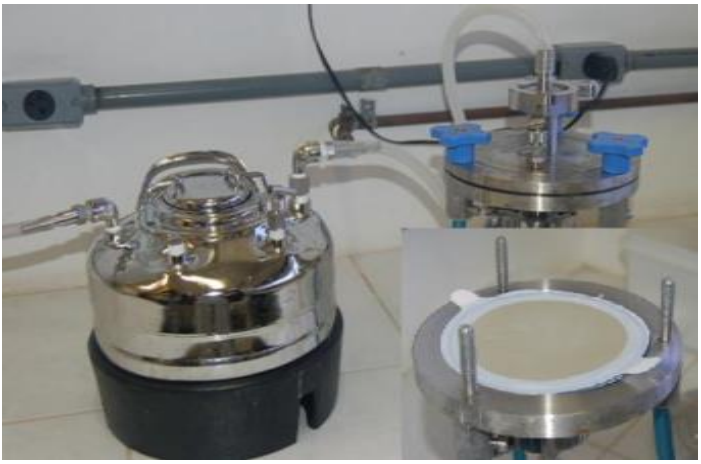
For sediment samples, the total solid, volatile solid, fixed solid and humidity were measured, according to APHA (2012). (These analyzes were carried out in partnership with the Water Ecology Laboratory Continental, Department of Ecology and Zoology at the Federal University of Santa Catarina, coordinated by Prof. Dr. Mauricio Mello Petrucio).

2.4 Viral concentration in water samples

Determination of viral concentrations in the surface water samples was performed using the protocol described by KATAYAMA et al., (2002). Briefly, water samples were filtered in a vacuum system provided with negatively charged membrane (Nihon Millipore®, Tokyo, Japan) with a pore size of 0.45 µm and a diameter of 142 mm (Fig. 14), with a flow of 200 mL / min. Prior to filtration, the pH was adjusted to 7.5-8.0 with the addition of 25 mL of $MgCl_2$ [2M] so, the viral particles, naturally negatively charged, were turned positive after the addition of the cationic Mg^{2+} ions, allowing the adsorption of these positively charged viral particles on the negatively charged membranes during the filtration process. The membrane was further washed with 350 mL of H_2SO_4 solution [0.5 mM] pH 3.0 to ensure retention of viral particles in it and remove other biosolids present. Then, the membrane was removed from the filter and, in sterile glassware; the viruses were eluted, by the

addition of 11 mL of NaOH [1 mM] pH 10.5, kept in constant contact with the membrane by stirring during 10 min. The eluate was neutralized by adding 50 µL of H_2SO_4 solution [50 mM] and 50 µL Tris-EDTA buffer solution [100X] 1 mM pH 8.0. After this step, the samples were ultra-filtrated by using a Centriprep Concentrator 50® system (Nihon Millipore®, Tokyo, Japan) to obtain a final volume of approximately 5.0 mL. The concentrated samples were stored at −80°C until further analysis.

Figure 14: Vacuum filtration system with a capacity of 5L water, used for viral concentration.



Source: Laboratory of Applied Virology (LVA-UFSC)

2.5 Viral concentration from sediment samples

Determination of viral concentrations in the sediment samples was performed according to the Environmental Protection Agency (EPA, 1992) guidelines, with minor modifications as described by SCHLINDWEIN et al., (2010).

Briefly, 0.05 M $AlCl_3$ solution was added to 20g of wet sediment diluted with the addition of an equal volume of phosphate buffer (PBS), and the pH was adjusted to 3.5 with 5 M HCl. To dislodge viruses, samples were then subjected to sonication on ice 3 times, each for 30 sec. followed by a centrifugation step at 2,422 χg for 15 min. at 4 ºC. The resulting pellet was suspended in 10 mL Glycine buffer 0.25M, pH 9.5 and stirred during 1 hr, followed by another centrifugation step at 7,656 χg for 30 min. at 4ºC. For viral concentration, PEG 6000 precipitation was employed as described by LEWIS; METCALF (1988).

The supernatant was transferred to another tube containing 8% PEG 6000, pH 7.2 and stay on the stirring for about 2 h 4ºC. A final centrifugation step was done at 7,656 χg for 90 min. at 4ºC. The pellet was suspended in 10.0 mL of 0.1 M PBS (pH 7.2). Decontamination step was carried out adding chloroform (1:3) followed by centrifugation at 7,656 χg for 15 min. The upper (aqueous) phase was recovered, aliquoted and stored at −80°C until further analysis.

2.6 Evaluation of viral recovery in surface water samples by adsorption-elution method

Water samples from the sampling sites were artificially inoculated with a known amount of the HAdV ($2x10^6$ PFU/mL). The samples were concentrated as described above and the viral recovery was determined by plaque assay and also by integrated cell culture RT-qPCR reaction, preceded by enzymatic treatment (ICC-et- RT-qPCR) (FONGARO et al., 2012).

2.7 Evaluation of viral recovery in sediment samples using Glycine/PEG buffer method compared with beef extract method

This experiment was designed to determine the efficiency of virus recovery after the concentration of the samples using the methods described by SCHLINDWEIN et al., (2010) and KATZENELSON et al., (1976) as described in section 2.5 and to decide which one would be used for the field samples, according to their performances. Two experiments were conducted in duplicate to evaluate the viral recovery rates from sediment samples. Murine Norovirus (MNV-1) and Simian Rotavirus (RV-SA11) were used as model viruses to seed the samples. Sediment samples from the sampling sites were artificially inoculated with a known amount of these viruses ($2.6x10^9$ GC/mL for MNV and $8.0x10^6$ GC/mL for RV-SA11). The samples were concentrated separately by Glycine/Polyethylene buffer as described in section 2.5 and beef extract method which was done briefly as following: sediment sample (300g) diluted in beef extract 3%, and then stirring step was done for 30 min. to form flocs. After a centrifugation step at 1,914 χg during 30 min. at 4ºC the supernatant was recovered and the pH was adjusted to 3.5 using 5M HCl and stirred again for more 30 min. Then a centrifugation step at 1914 χg for 15 min. at 4ºC was carried out and the pellet was suspended in 10 mL of 0.1 M phosphate buffer (pH 9). Decontamination step was carried out as described above in section 2.5 using chloroform. Then, MNV and SA-11 RV recovery from the respective samples was quantified by real-time RT-PCR using a

TaqMan technique, as previously described by BAERT, et al., (2008) and ZENG et al., (2008). The calculation of the RVA-SA11 recovery percentage (in number of genome copies) was accounted for the original number of viruses seeded in the experiment.

2.8 Extraction of viral nucleic acids

Nucleic acid extraction was performed using a QIAamp MinElute® Virus Spin Kit (Qiagen, Brazil), following the manufacturer's instructions. Briefly, the QIAamp MinElute Virus Spin Kit simplifies purification of viral DNA and RNA with fast spin-column filled with a silica-based reagent based on the protocol already described by BOOM et al., (1990). The starting sample volumes were 0.2 mL and this technique combines the selective binding properties of the nucleic acids to the silica columns with a flexible elution volumes of around 60 µL followed by storing at -80 °C prior to analysis.

2.9 Quantitative PCR assay (qPCR)

Real time quantitative PCR (qPCR) was performed as described by HERNROTH et al., (2002); JOTHIKUMAR et al., (2005); ZENG et al., (2008) and Baert et al., (2008) for amplification of HAdV, HAV, RVA and MNV, respectively, using primers and probes as shown in Table 1. The Taqman Universal qPCR *Master Mix* (Applied Biosystems) was used for HAdV and the Kit QuantiTect probe RT-PCR (QIAGEN) was used for HAV, RVA, and MNV.

All amplifications were performed in a StepOne Plus® Real-Time qPCR System (Applied Biosystems). Each sample was analyzed in duplicate. To avoid inhibition of the enzymatic assay, the nucleic acids were prior diluted at 1:10 before their addition on the qPCR reaction. A standard curve was generated for all of the viruses using a 10-fold serial dilution of the pCR2.1 vector (Invitrogen, USA) containing the target region appropriate for the virus type. Triplicate samples were used for each dilution point, and a standard curve was run for each set of assays. Ultrapure water was used as the non-template control, and non-amplification controls were included in each run.

Table 1: Primers and probes used for detection of genomes copies of HAdV, HAV, RVA, and MNV

Virus	Primers and probes	Sequence 5´-3´	Final conc. of Primers	Ref.
HAdV	HAdV F	C(AT)TACATGCACATC(GT)C(CG)GG	900 nM	Hemroth *et al.*
	HAdV R	C(AG)CGGGC(GA)AA(CT)TGCACCAG	900 nM	(2002)
	HAdV probe	**FAM**-CCGGGCTCAGGTACTCCGAGGCGTCCT-**TAMRA**	225 nM	
RVA	RVA F	ACCATCTWCACRTRACCCTCTATGAG	0.25 µM	Zeng *et al.* (2008)
	RVA R	GGTCACATAACGCCCCTATAGC	0.25 µM	
	RVA probe	**VIC**-AGTTAAAAGCTAACACTGTCAAA-**MGB**	0.15 µM	
HAV	HAV F	GGTAGGCTACGGGTGAAAC	0.25 µM	Jothikumar *et al.*
	HAV R	GCGGATATTGGTGAGTTGTT	0.25 µM	(2005)
	HAV probe	**FAM**-CTTAGGCTAATACTTTATGAAGAGATGC-**TAMRA**	0.15 µM	
MNV	MNV-F	CAC GCC ACC GAT CTG TTC TG	0.25 µM	Baert et al., (2008)
	MNV-R	GCG CTG CGC CAT CAC TC	0.25 µM	
	MNV probe	**FAM**- CGC TTT GGA ACA ATG –MGB– **NFQ**	0.15 µM	

2.10 Viral integrity test

This study aimed to identify intact adenovirus particles (HAdV) through enzymatic assay in order to degrade possible free viral genomes present in the sample matrix and compare the total genomes detected with the genomes from intact viral particles.

To study the presence of undamaged HAdV particles, the samples were treated with DNAse I. Briefly, the reaction was performed by adding 1 U of DNAse I (a sufficient quantity to degrade 100% of DNA added), 1X buffer and 170 µL of nuclease-free water as a negative control for each concentrated sample, and the reaction was then incubated for 15 min at room temperature. Then, the enzyme activity was inactivated by adding EDTA at final concentration of 25 mM and incubation for 10 min. at 70°C. This was done to check the integrity of the viral capsid, because genetic material that was not protected by the viral capsid would be degraded by this nuclease (NUANUALSUWAN; CLIVER, 2002). All samples positive for HAdV in the real time PCR assay were subjected to a DNAse I degradation reaction following the manufacturer's instructions (Sigma–Aldrich, Steinheim, Germany) to degrade all free viral DNA present in the samples, as described by VIANCELLI et al., (2012). All treated samples were subjected to nucleic acid extraction and qPCR, as described in section 2.9.

2.11 Study of the infectivity of HAdV in surface water and sediment samples

This study aimed to identify infectious/viable particles of HAdV that were previously detected by qPCR, by inoculating samples in cell culture and perform the plaque assay which was used to assess the viral infectivity.

2.11.1 Cell Line

The cultivation of the cell line A549 (epithelial cells derived from human lung carcinoma and permissive to most human adenoviruses kindly provided by Dr. Rosina Gironès from the University of Barcelona, Spain) was carried out in cell culture flasks ($75cm^2$), using minimal essential medium containing Eagle's salts (MEM) supplemented with 5% fetal bovine serum [(FBS) Gibco], 1% antibiotics and antifungal [(PSA-penicillin G Cultilab 100U/mL/100 g streptomycin sulfate/mL /Amphotericin B 0.25 g / mL.

2.11.2 Cytotoxicity assay

The cytotoxicity of concentrated surface water and sediment samples were evaluated on A549 cells. These cells were propagated in cell culture using 24-well plates at a density of 2.5×10^5 cells per well, as described on section 2.12, but without addition of FBS, 24 h before conducting the tests.

Environmental samples concentrated as described section 2.4 and 2.5, were pretreated with 1% antibiotic/antifungal (100 U penicillin G / mL / streptomycin 100µg /mL amphotericin B 0.25µg /mL) and diluted in 1X MEM in the following proportions: 1:1, 1:2, 1:4. An inoculum of 100 µL of each dilution in triplicate were placed in contact with the cells for 1h at 37 °C in 5% CO_2 atmosphere and uniform mixing every 15 min. Subsequently, the inoculum was removed and 1 mL of MEM containing 1% PSA was added to the cells, two cell controls were maintained and these cells contained only medium. The plates were incubated at 37°C in 5% CO_2 atmosphere and the cell monolayer was monitored for seven days.

Cells containing inoculated test samples were compared with control cell under a light microscope. Sequentially, cells were fixed and stained with 300µL of Naphthalene Black solution (0.1% Naphthalene Black, 5% Acetic Acid, pH 2.3-2.4). The cell monolayer was stained and examined for the non-cytotoxic dilution, for each of tested samples in order to be furthered used for infectivity assays.

2.11.3 Plaque Assay for HAdV

To quantify the presence of infectious HAdV in environmental samples, all of the water and sediment samples testing positive for viral genome presence (as previously detected by qPCR) were treated with antibiotics (10 U/mL penicillin, 10 μg/ mL streptomycin and 0.025 μg /mL amphotericin B). For all tested samples, a non-cytotoxic dilution was then selected and inoculated (0.2 mL) into A549 cells, in triplicate. These cells were previously cultivated in 24-well tissue culture plates at a density of 3.0×10^6 cells / well and were incubated at 37°C in 5% CO_2 for 24 h as described by FONGARO et al., (2013); CROMEANS et al., (2008); and RIGOTTO et al., (2010).

Briefly, the cells were incubated during 1h at 37°C in 5% CO_2 and were gently rocked every 15 min for viral adsorption. The inoculated cells were then carefully washed once with pre-warmed PBS and overlaid with warm high glucose Dulbecco's modified Eagle's medium (2x), 0.6% warm Bacto-agar containing 5% FBS, 0.1 mM sodium pyruvate, 10 U/mL penicillin, 10 μg/mL streptomycin and 26 mM $MgCl_2$. The plates were incubated at 37°C in 5% CO_2 for 7 days. One week post-infection, the agar overlay was removed gently, and the cells were stained with 20% Gram's crystal violet. The cell monolayer was examined microscopically, and plaques were counted and expressed as PFU/mL.

2.12 Statistical analyses

To evaluate the possible correlation between viral detection in samples from each point of collection, a Pearson correlation and linear regression test, a two way ANOVA test and a Student's t test were performed using GraphPad Prism 5.0 (USA); data were considered statistically significant at a *P*-value ≤ 0.05.

3. RESULTS

3.1 Physical-chemical analysis

The means of the physical-chemical parameters measured for all samples from each collection point in both summer and winter collections are presented in Table 2. According to these data, the averages of sample temperature, pH, salinity, and conductivity during summer and winter, were not statistically significant ($P>0.05$), while the mean dissolved oxygen for each point of collection during the summer and winter was statistically significant among points of collection ($P\leq0.01$).

Regarding the sediment analysis, the averages of the total solids, fixed solids, volatile solids and humidity of the samples were not statistically significant among the points of collection either in the summer or winter periods (P>0.05), as shown in Table 2. As we can observe on Fig. 15, the percent of dissolved oxygen at each point was different but this difference during summer and winter was not statistically significant (P>0.01).

Figure 15: Percent average of dissolved Oxygen in each point of collection

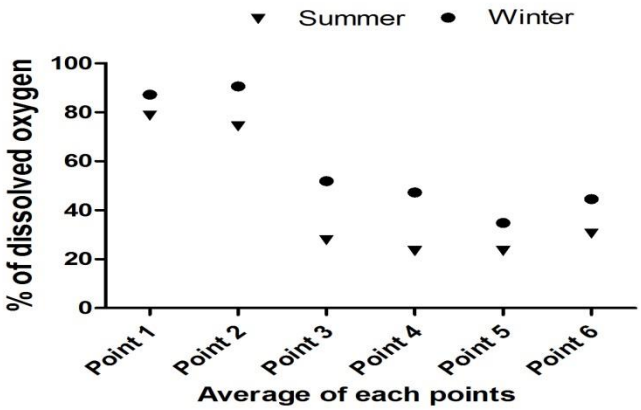


Table 2: Physical-chemical analysis for surface water and sediment analysis in each point of collection during the summer (sum.) and winter (win.) seasons, statistically significant differences noted by (*), (#) SD= Standard deviation.

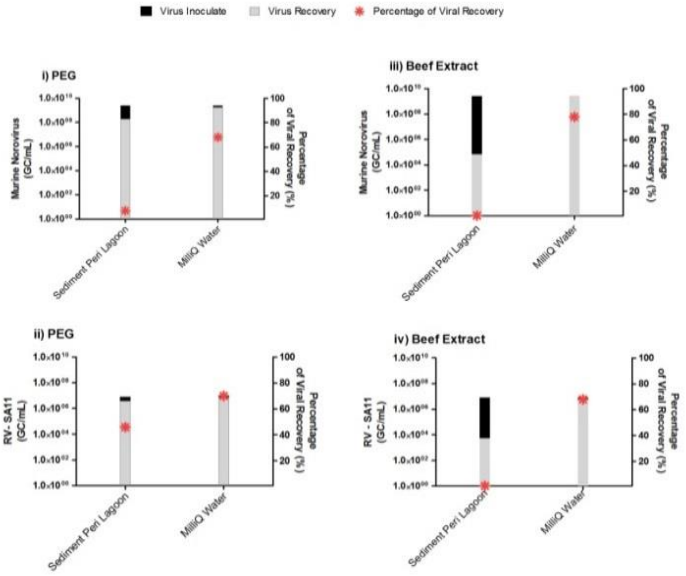
Parameter	Point 1		Point 2		Point 3		Point 4		Point 5		Point 6	
	Sum.	Win.	Sum.	Win.	Sum.	Win.	Sum.	Win.	Sum.	Win.	Sum.	Win.
Surface water												
Sample temp. (°C)	24.95 ±1.00	18.75 ±1.46	24.88 ±1.62	17.78 ±1.39	24.75 ±1.94	18.83 ±2.60	24.58 ±2.08	18.25 ±2.06	25.18 ±2.88	18.13 ±2.62	26.2 ±3.52	18.78 ±2.4
pH	6.05 ±0.04	6.28 ±0.24	6.66 ±0.16	6.25 ±0.34	6.18 ±0.30	6.48 ±0.26	6.4 ±0.21	6.58 ±0.35	6.75 ±0.15	6.33 ±0.39	6.25 ±0.43	6.45 ±0.34
Conductivity (µS/cm)	39.88 ±5.02	43.85 ±10.9	38.7 ±5.91	47.9 ±12.7	46.8 ±19.69	46.1 ±12.6	49.5 ±15.35	46.93 ±20.1	54 ±11.66	41 ±15.7	43.28 ±20.52	42.92 ±10.11
Dissolved Oxygen (mg/L)	6.75* ±0.96	7.98* ±0.39	5.75* ±2.62	8.71* ±3.77	2.24* ±0.46	4.82* ±0.38	1.89* ±0.41	4.61* ±0.35	1.85* ±0.47	3.89* ±0.55	2.76* ±1.46	6.7* ±4.0
Salinity (ppt)	-		-		-		-		0.1 ±0.07	0.73 ±0.82	0.37 ±0.3	0.75 ±0.83
Sediment												
Total Solid (mg/L)	72.84#		122.25		119.07		121.61		98.8#		135.36	
Fixed solid (mg/L)	98.6#		165.51		162.20		165.45		134.38#		184.11	
Volatile solid (mg/L)	1.35#		2.26		3.71		3.77		3.06#		4.19	
Humidity (%)	27#		45		44		44.7		36.3#		49.7	

± Standard deviation

3.2 Viral recovery assay for water and sediment samples

The HAdV recovery rate, as determined by FONGARO et al., (2013) using qPCR (GC units), was approximately 10% (total of 8 × 105 GC/mL) for water samples. For sediment experiment; the recovery of MNV was 7.7% (total of 2.02 × 10^8 GC/mL) for sediment and 68% (total of 1.77 × 10^9 GC/mL) for MilliQ water using Glycine/PEG method described by SCHLINDWEIN et al., (2010) and 0.002% (total of **7** × 10^4 GC/mL) for sediment and 87% for MilliQ water (total of 2.3 × 10^9 GC/mL) using the beef extract method described by KATZENELSON et al., (1976). For RV-SA11 the recovery using Glycine/PEG method was 46% (total of 3.7 × 10^6 GC/mL) for sediment and 70% (total of 5.6 × 10^6 GC/mL) for MilliQ water, while using the beef extract method was 0.06% (total of 5.3 × 10^3 GC/mL) for sediment and 68% (total of 5.4 × 10^6 GC/mL) for MilliQ water as shown in Fig. 16.

Figure 16: Recovery rate for MNV-1 and RV-SA11 using two concentration methods for viruses in sediment and MilliQ water.

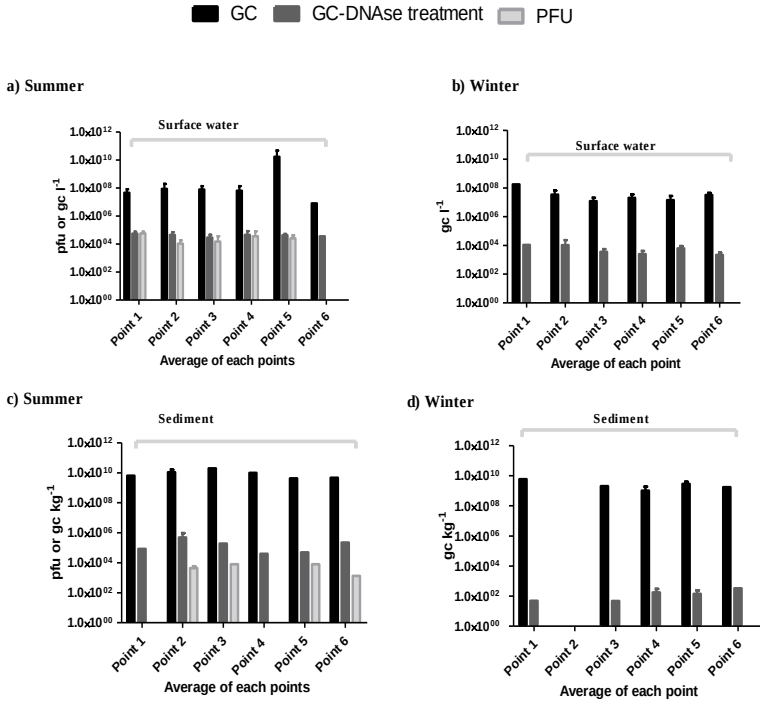


3.3 Viral analysis

For surface waters collected during the summer, the incidence of HAdV was 70.8% (17/24), ranging from 6.9 x 10^5 to 2.4 x 10^8 GC/L. All 17 positive samples were shown to have undamaged particles after nuclease treatment, in concentrations ranging from 1.2 x 10^4 to 8.9 x 10^4 GC/L, and from these, 82.4% (14/17) contained infectious particles, in concentrations ranging from 4.0 x 10^3 to 1.07 x 10^4 PFU/L. In winter collections, the HAdV incidence was 62.5% (15/24), ranging from 8.9 x 10^5 to 1.7 x 10^8 GC/L. Of these positive samples, 93.3% (14/15) of samples had undamaged particles after nuclease treatment, in concentrations ranging from 1.3 x 10^3 to 2.5 x 10^4 GC/L. All of these undamaged viruses were infectious by plaque assay. The mean results for HAdV GC for each point were statistically significant for both the summer and winter collections (P<0.05), as shown in Fig. 17 (a,b).

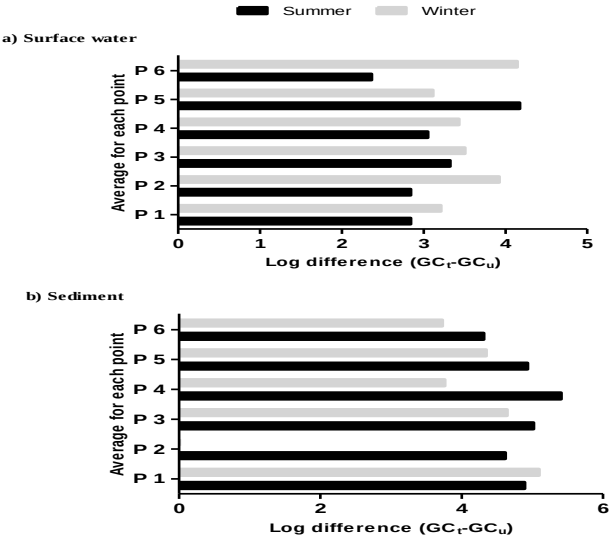
For sediment samples collected in the summer, the HAdV incidence was 37.5% (9/24), ranging from 4.2 x 10^9 to 1.9 x 10^{10} GC/Kg. All positive samples were shown to have undamaged particles after nuclease treatment, in concentrations ranging from 1.8 x 10^4 to 8.6 x 10^5 GC/L. Of these samples, 66.7% (6/9) contained infectious HAdV, in concentrations ranging from 1.3 x 10^3 to 8 x 10^4 PFU/Kg. The results of winter collection revealed a HAdV incidence of 37.5% (9/24), ranging from 3.1 x 10^8 to 6.0 x 10^9 GC/Kg, and 100% (9/9) undamaged particles ranging in concentration from 4.7 x 10^4 to 3.3 x 10^5 GC/L. No infectious samples were detected by PFU. These results were statistically significant (P<0.05), as shown in Fig. 17 (c, d).

Figure 17 : Average number of HAdV genome copies before and after treatment with DNase I and the number of infectious particles in surface water and sediment samples during summer and winter in Peri Lagoon and the Sangradouro River (n=24 for surface water and n=24 for sediment)



The difference between the total genome and the intact HAdV was statistically significant (P<0.05) in all cases. The log difference was performed by: [$\log_{10}$ ($GC_{total}/GC_{undamaged}$)]. The general mean of the log difference in surface water samples during summer and winter was 0.81±0.56 and in sediment samples was 1.27±1.59, as shown in Fig. 18. This log reduction was calculated using the following formula: $\log_{10}$ reduction = $\log_{10}$ (GC_t/GC_u), where GC_t is the total number of HAdV particles (GC/L) and GC_u is the number of undamaged HAdV particles (GC/L) (P<0.05).

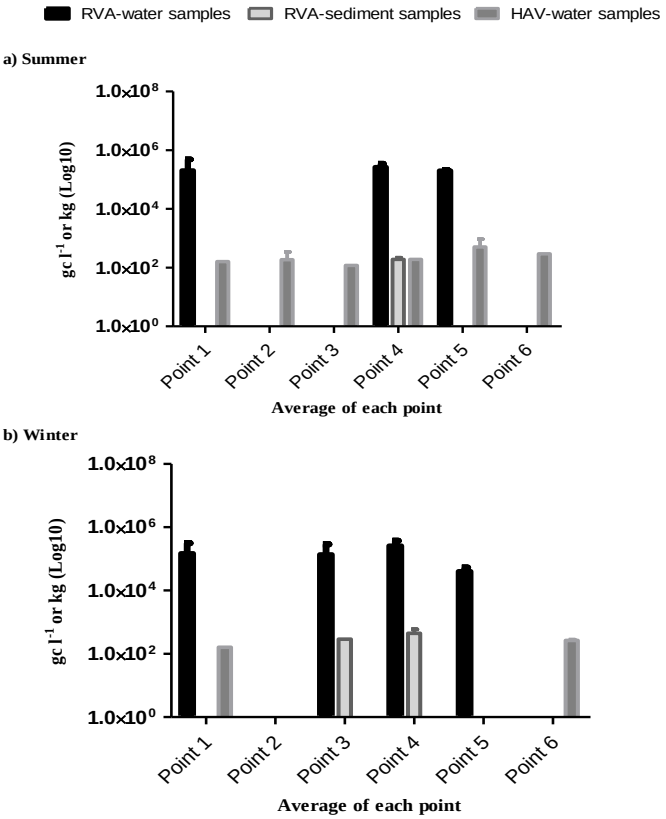
Figure 18: Log difference [$\log_{10}$ ($GC_{total}/GC_{undamaged}$)] between total and undamaged viral GC during summer and winter collections ($P<0.05$).



The RVA incidence in surface water samples collected during summer was 20.8% (5/24), ranging from 1.94×10^5 to 4.1×10^5 GC/L. In winter, this incidence was 45.8% (11/24), ranging from 3.1×10^4 to 5.4×10^5 GC/L. For sediment samples collected during summer, the RVA incidence was 8.3% (2/24), ranging from 1.73×10^5 to 2.1×10^5 GC/Kg. In winter, this incidence was 12.5% (3/24), ranging from 2.9×10^5 3.4×10^5 GC/Kg as shown in Fig. 19.

The HAV incidence in water samples was 45.8% (11/24), ranging from 5.5×10^1 1.1×10^3 GC/L and 12.5% (3/24), ranging from 1.6×10^2 to 2.7×10^2 GC/L for the summer and winter collections, respectively. Figure 19 (a and b) illustrate the RVA and HAV incidences for all points of collection during the summer and winter collections.

Figure 19: Average number of RVA and HAV GC occurring in surface water and sediment samples during summer and winter at the six points of collection (n=24 for surface water and sediment) (P>0.05).



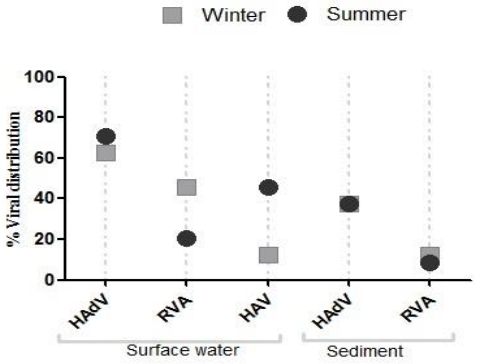
The overall mean percentages of enteric viruses detected in our study in surface water and sediment samples from summer and winter collections showed that HAdV was the most frequently detected enteric virus, followed by RVA and then HAV, as shown in Table 3.

Table 3: Prevalence of HAdV, RVA, and HAV in surface water (wat.) and sediment (sed.) samples for each point of collection during the summer and winter seasons.

Points of collection		HAdV	RVA	HAV
Point 1	Wat.	4/8 (50%)	4/8 (50%)	2/8 (25%)
	Sed.	2/8 (25%)		
Point 2	Wat.	7/8 (88%)	ND	3/8 (38%)
	Sed.	4/8 (50%)		
Point 3	Wat.	6/8 (75%)	3/8 (38%)	1/8 (13%)
	Sed.	2/8 (25%)	1/8 (13%)	
Point 4	Wat.	5/8 (63%)	4/8 (50%)	1/8 (13%)
	Sed.	4/8 (50%)	4/8 (50%)	
Point 5	Wat.	6/8 (75%)	4/8 (50%)	4/8 (50%)
	Sed.	4/8 (50%)		
Point 6	Wat.	4/8 (50%)	ND	3/8 (38%)
	Sed.	2/8 (25%)		

As shown in Fig. 20, the overall mean percentages regarding the seasonal occurrence of the three viruses' shows that RVA was more frequently detected in both surface water and sediment during the winter season. In contrast, the frequency of HAV detected in summer samples was higher than in winter samples, but only for surface water. HAdV was the most prevalent virus during summer and winter and showed no difference in seasonal prevalence. However, the seasonal distributions of these viruses were not statistically significant ($P>0.05$).

Figure 20: Viral distribution (percentages) in surface water and sediment samples during summer and winter collections ($P>0.05$).



CHAPTER II

DISTRIBUTION AND VERTICAL ABUNDANCE OF VIRUSES IN A TROPICAL LAGOON USED AS WATER SOURCE FOR DRINKING WATER AND SEDIMENT IN SOUTHERN BRAZIL

CHAPTER II

1. INTRODUCTION

This chapter will present and discuss the results obtained during the second year of collection (2014) from the water sources (Peri Lagoon) and different depths of water column by real time PCR and also by cell culture technique for the detection of the human viruses and somatic coliphages. Two liters (2 L each) of water and 20 g of fluffy sediment samples were collected monthly in each of the pre-selected locations as further described. These results are under revision in International Journal of Hygiene and Environmental Health.

International Journal of Hygiene and Environmental Health

Spatial distribution of enteric viruses in a Lagoon used as drinking water source and recreation in Southern Brazil

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2. MATERIAL AND METHODS

2.1 Description of study area

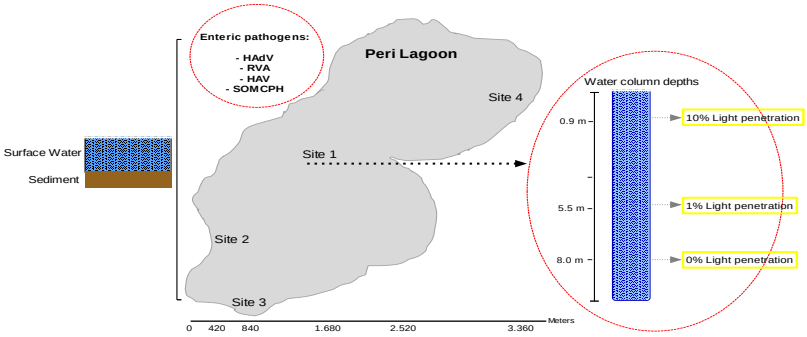
As described before in Chapter I Peri Lagoon is fed mainly by two waterfalls: Cachoeira Grande and Ribeirão Grande; this Lagoon is widely used for recreational activities (swimming and fishing) and stands out for participating in the System East-Coast-South Water Treatment Plant, supplying about 110,000 inhabitants of Florianópolis in the districts of Barra da Lagoa, Lagoa da Conceição, Campeche, Morro das Pedras, Armação and Ribeirão da Ilha.

2.2 Sampling

Due to its importance we selected four points in Peri Lagoon for water and sediment sampling collections as collection sites 1) located at the center of Lagoon and this site was composed by 4 collection points of water samples [surface water (100% light penetration), 0.9 meter in deep (10% light penetration), 5.5 m in deep (1% light penetration), 8 m in deep (0% light penetration)], and one sediment sample as shown in Fig. 22; site 2) located in waterfalls Cachoeira Grande as shown in Fig. 23; site 3) located in waterfalls Ribeirão Grande as shown in Fig. 24; site 4) located in the beach of Lagoon as shown in Fig. 25;. Surface water and sediment samples were collected monthly from January 2014 until December 2014, 7 water samples (include: 4 samples collected from surface water in different sites and 3 water samples collected from different depth of water column), and 4 sediment samples / collection, totaling 132 water and sediment samples). As shown in Fig. 21. Sediment samples were collected using ***Petersen*** grab samplers as mentioned before in Chapter I.

Note: The collection of the samples at Peri Lagoon were possible due to a partnership established with Continental Water Ecology Laboratory from UFSC (Federal University of Santa Catarina), coordinated by Prof. Dr. Mauricio Mello Petrucio, who we thank very much for this important collaboration.

Figure 21: Location of Peri lagoon and the sampled sites. Numbers indicate the sampling sites. The column represents the different depths of the water column sampling.



Source: Author (article 2)

Figure 22: Site 1 of collection, center of Peri Lagoon, with collection in surface water (point 1), 0.9 meter in deep (point 2), 5.5 m in deep (point 3) and 8 m in deep (point 4)

Figure 23: Site 2 of collection, waterfalls Cachoeira Grande

Figure 24: Site 3 of collection, waterfalls Ribeirão Grande

Figure: 25: Site 4 of collection, beach of Peri Lagoon

2.3 Evaluation of physical-chemical parameters and nutrients for water samples

The evaluations of the physical-chemical parameters were taken in four micro-environments (MAs) of Peri Lagoon. For this, in each collection, the following physical-chemical parameters were assessed *in situ* by means of Multiparameter probe (YSI-85): water temperature (TA), conductivity (Cond.), pH and dissolved oxygen (DO). For the analyzes of nutrients were done only for samples taken from site 1 (center of Lagoon), water samples from each collection site, were taken to the laboratory to determine the amount of nitrite ($N.NO_2$-) (GOLTERMAN et al., 1978), nitrate ($N.NO_3$-) (MACKERETH et al., 1978), ammonia ($N.NH_4$ +) (KOROLEFF, 1976) and for phosphorus (these analyzes were carried out in partnership with the Laboratory of Continental Water Ecology, Department of Ecology and Zoology at Federal University of Santa Catarina, under the coordination of Prof. Dr. Mauricio Mello Petrucio).

2.4 Coliphage propagation

Typified strains of somatic coliphages used as positive controls in this study: PhiX174 (*Microviridae*), phages and their respective bacterial hosts were provided by Prof. Dr. Maria Elisa Magri (UFSC, Dept. of Environmental and Sanitary Engineering).

2.5 Detection and enumeration of Bacteriophage somatic coliphage in surface water and sediment samples

Bacteriophages were quantified by a double agar layer technique following the ISO 10705-2 standard (ISO 2000) for enumeration of somatic coliphages (SOMCPH). The volume of water tested for each phage was 10 mL. The detection limit (DL) as detected by serial dilution for somatic coliphage was 10 PFU/100 mL. *E. coli* ATCC 13706 was used as the host for somatic coliphage detection according to ISO/FDIS 10705-2. Briefly, the water samples were filtered through a cellulose ester membrane filter, with 0.22 µm pore size and 47 mm diameter. In case of sediment samples, they were diluted first with peptone water (1:10) and then filtered through a cellulose ester membrane filter. *E. coli* C strain ATCC 13706 were grown in modified Scholten's broth (MSB) was used in the quantification of somatic coliphages. To each culture tube, 1 mL of the original water sample (diluted in case of sediment) was added to 1 mL of inoculum culture and 2.5 mL of semi solid Modified Scholtens Agar (ssMSA) heated at 47°C. The solution was mixed carefully avoiding the formation of air bubbles and poured on a layer of complete solid Modified Scholtens Agar (MSA) prepared previously in a 9 cm Petri dish pre-warmed at room temperature. After solidification on a horizontal, cool surface, the plates were incubated upside-down at 36±2 ºC for 18±2 h. The results were expressed in PFU/L.

2.6 Viral Concentration

2.6.1 W**ater samples**

Viral concentrations in the surface water samples were performed using the protocol described by KATAYAMA et al., (2002) as described in chapter I.

2.6.2 Sediment samples

Viral concentrations in the sediment samples were performed according to the Environmental Protection Agency (EPA, 1992) guidelines, with minor modifications as described by SCHLINDWEIN et al. (2010) as described in chapter I.

2.7 Extraction of viral nucleic acids

Nucleic acid extractions were performed using a QIAmp MinElute Virus Spin Kit (Qiagen), following the manufacturer's instructions as described in in chapter I.

2.8 Quantitative PCR assay (qPCR)

Real time quantitative PCR (qPCR) was performed as described in Chapter I and according to HERNROTH et al., (2002); JOTHIKUMAR et al., (2005); and ZENG et al., (2008) for amplification of HAdV, HAV and RVA.

2.9 Statistical analyses

To evaluate the possible correlation between viral detection in samples from each point of collection, a Pearson correlation and linear regression test, a two way ANOVA test and a Student's t test were performed using GraphPad Prism 5.0 (USA); data were considered statistically significant at a *P*-value ≤ 0.05.

3. RESULTS

3.1 Evaluation of physical-chemical parameters and nutrients

Surface water samples from the Peri Lagoon area were assessed monthly over a year, for the following physical-chemical parameters: water temperature, conductivity [Cond.], pH and dissolved oxygen [DO] and nutrients for site 1, Center of Peri Lagoon [Ortho-Phosphate, Total Phosphate, nitrite ($N.NO_2$-), nitrate ($N.NO_3$-), ammonia ($N.NH_4$ +), and total Nitrogen (TN).

The means of the physical-chemical parameters measured for all samples from each collection point along one year collections are presented in Table 4 and as shown in Fig. 26 (a, b, c, d, e, f, g, h, i, and j) and in Fig. 27 (a, b, c, and d).

According to the complete analysis of these parameters, no one showed any significant spatial or temporal variation, except DO. For this parameter, in the collections of site 1 at different depths, DO was correlated positively among each point at different depths [surface water, 0.9 meter in deep, 5.5 m in deep, 8 m in deep] and this correlation was statistically significant ($P \leq 0.0001$) (Pearson test-ANOVA).

Table 4: Physical-chemical analysis and nutrients for water in each point of collections, statistically significant differences noted by (*), (#)

Parameters	Site 1 Surface water Mean ± SD	Site 1 0.9 meter depth Mean ± SD	Site 1 5.5 meter depth Mean ± SD	Site 1 8 meter depth Mean ± SD	Site 2	Site 3	Site 4
Temp. of water (°C)	23.1±3.9	20.9±6.7	23.0±3.7	22.8±3.8	23.4±3.9	23.3±3.8	22.9±3.6
pH	7.1±0.8	7.0±0.8	7.1±0.8	7.1±0.7	7.1±0.8	7.1±0.9	7.1±0.7
Cond. (µs cm^{-1})	73.1± 21.4	73.5± 3.3	73.8± 3.4	74.2± 22.6	74.0± 3.3	74.1±3.6	73.6±3.1
DO (mg L^{-1})	8.0± 2.4*#	7.9± 0.6*#	7.3± 0.9*#	7.3± 0.8*#	7.8± 0.8#	7.7± 0.8#	7.9± 0.9#
OP (µg L^{-1})	2.08±0.87	2.01± 1.22	2.59± 0.71	2.24± 1.49	NT	NT	NT
TP(µg L^{-1})	9.57± 4.44	10.20± 3.51	10.73± 3.81	12.23± 5.05			
NO_2^-.N (µg L^{-1})	0.07± 0.05	0.15± 0.26	0.07± 0.07	0.08± 0.06	NT	NT	NT
NO_3^-.N (µg L^{-1})	4.45± 2.90	4.64± 3.15	4.84± 3.35	4.40± 2.93			
NH_4^+.N (µg L^{-1})	1.97± 1.59	2.51± 2.11	2.41± 1.69	2.85± 2.72	NT	NT	NT
N_2 (µg L^{-1})	447.8± 332.3	504.1± 326.6	508.0± 321.6	516.1± 283.3			

± Standard deviation, * statistically significant ($P \leq 0.0001$), # Positive correlation with Pearson test ($r^2=0.99$) for site 1 and ($r^2=0.88$) for sites 2, 3, 4. (NT= not tested).

Figure 26: Monthly quantification (Jan. / 2014 to Dec. / 2014) of the physical-chemical parameters and nutrients (a, b, c, d, e, f, g, h, i, j) for site 1 of collection

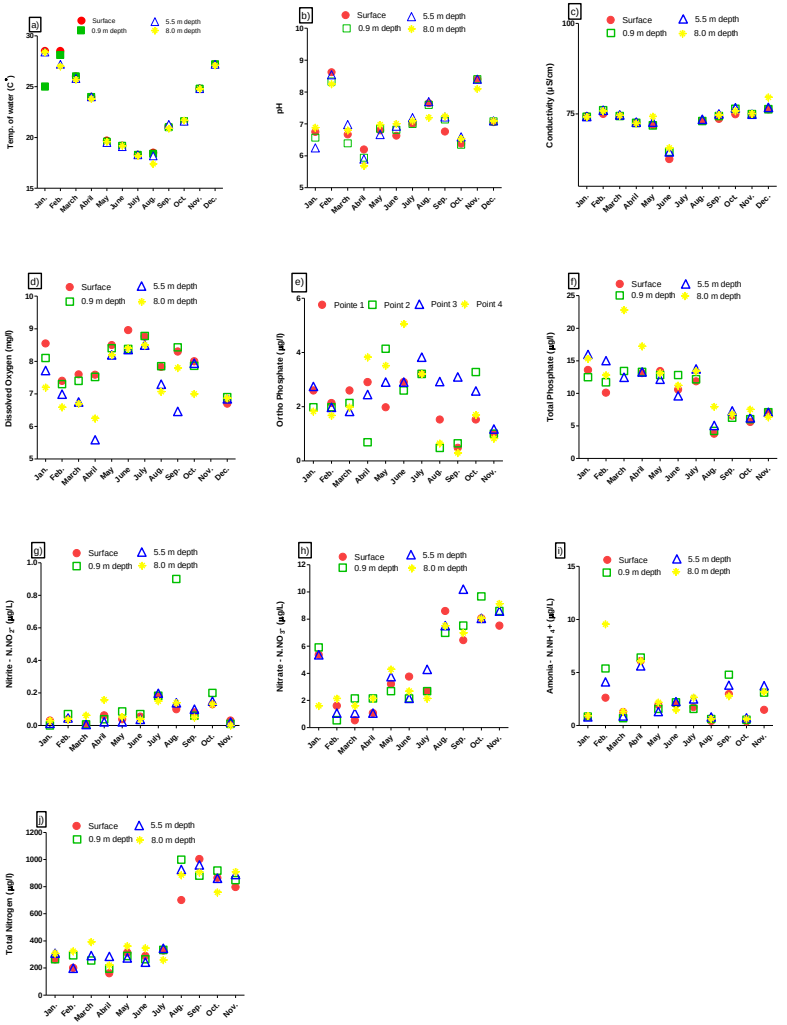
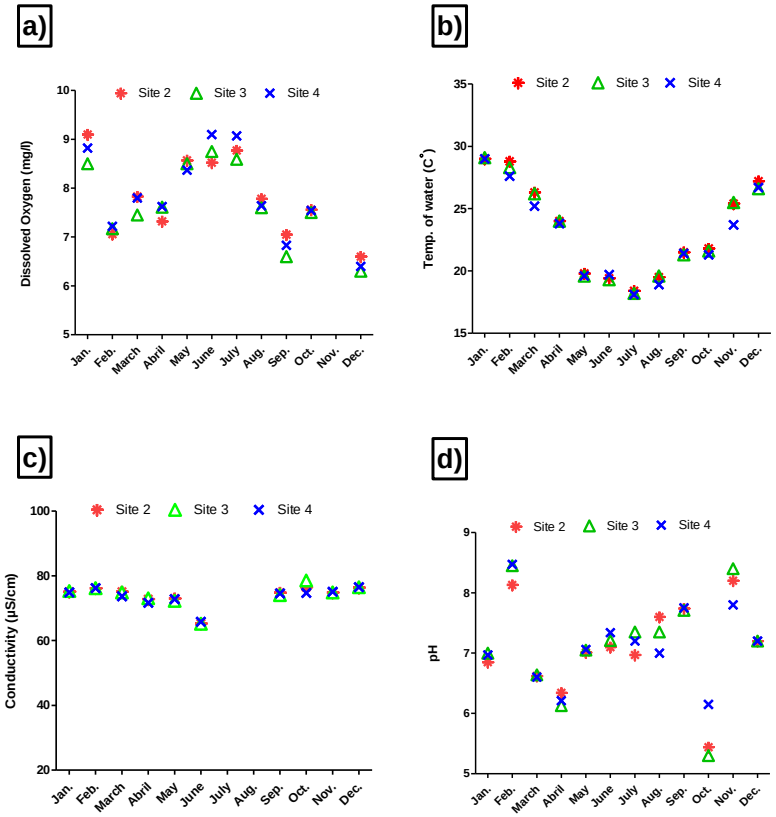


Figure 27: Monthly quantification (Jan. / 2014 to Dec. / 2014) of the physical-chemical parameters (a, b, c, and d) for sites of collection 2, 3, 4.



3.2 Detection and enumeration of somatic coliphages in water and sediment samples

Along one year of collection, 84 water samples and 48 sediment samples were collected monthly at Peri Lagoon. Infectious somatic coliphages were detected in 43% (36/84); while in sediment samples only 18.75% (9/48), contained infectious somatic coliphage and it was clear from the results that somatic coliphages were prevalent in winter and spring as shown in Fig. 28 (a, b) and Fig. 29.

Figure 28 (a, b): Detection and enumeration of somatic coliphages in water. In "a", different depths of collection in site 1 (center of Peri Lagoon at surface water [point 1], 0.9 meter in deep [point 2], 5.5 m in deep [point 3] and 8 m in deep [point 4]); "b", water samples from sites 2, 3 and 4.

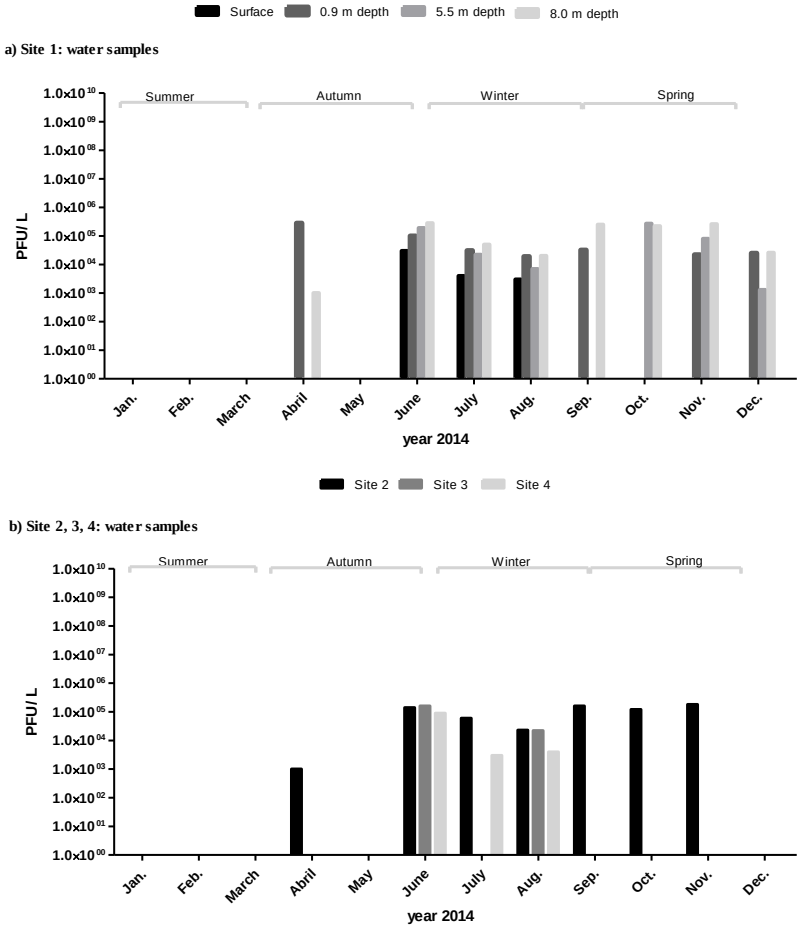
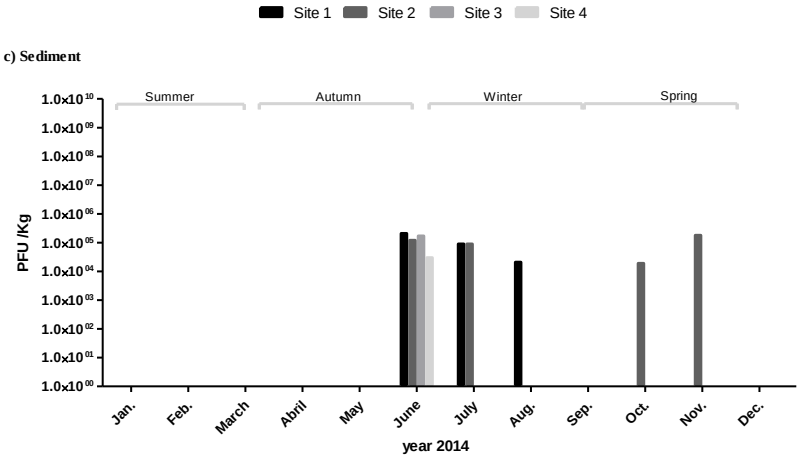


Figure 29: Detection and enumeration of somatic coliphages in sediment sample for all sites of collections



3.3 Detection of HAdV genome copies in water and sediment samples

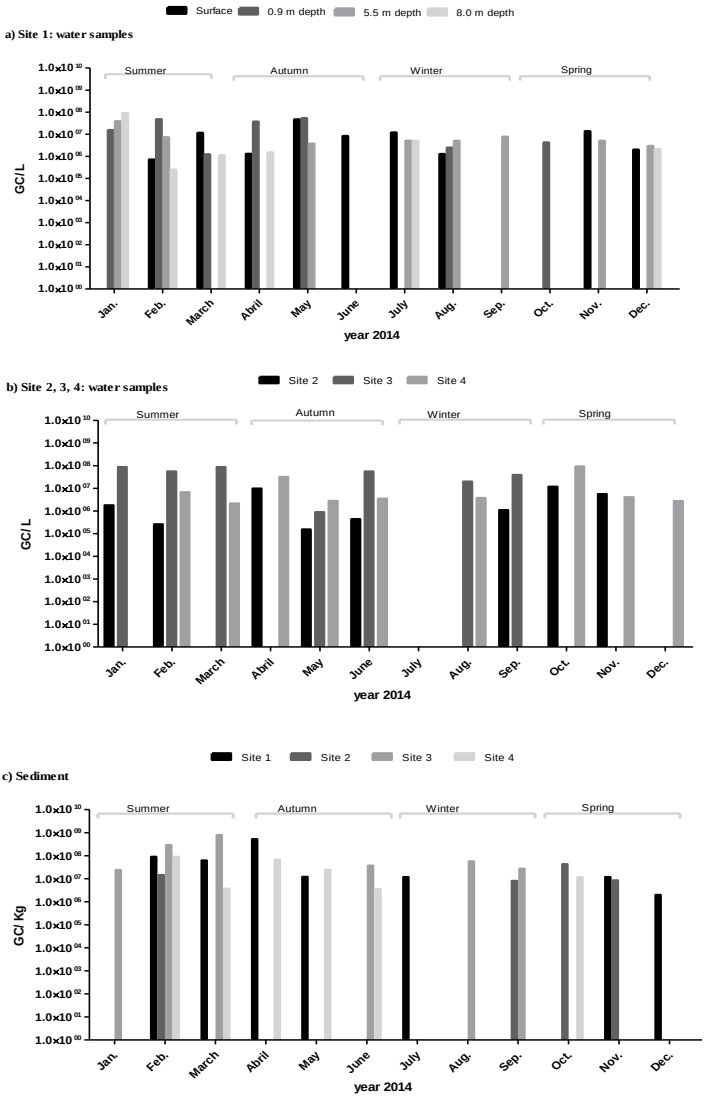
A sum of 84 water samples and 48 sediment samples were analyzed for the presence and quantification of HAdV genomes and divided as following: for water samples, 48 were collected in the center of Lagoon (site 1) divided by 12 samples for each depths ([surface], [0.9 meter], [5.5 meters], [8 meters]), 12 water samples were collected in site 2 (Cachoeira Grande), 12 water samples were collected in site 3 (Ribeirão Grande), and 12 water samples were collected in site 4 (beach of Peri Lagoon). HAdV GC was detected in 64.3% (54/84) of the samples. For sediment samples a total of 48 sediment samples were analyzed for the presence and quantification of HAdV genomes and divided as following: 12 samples were collected monthly in the center of Peri Lagoon (site 1), 12 samples were collected in site 2 (Cachoeira Grande), 12 samples were collected in site 3 (Ribeirão Grande), and 12 samples were collected in site 4 (beach of Peri Lagoon). HAdV GC was detected in 47.9% (23/48) as presented in details in Table 5, which summarizes the results obtained by quantitation of HAdV present in the collected samples, monitored for one year. The values correspond to the range of the number of genomic copies per liter of water (minimum and maximum values), as well as the overall average of viral genome copies in each collection site.

Table 5: HAdV genomic copies in water samples collected during one year of collection (minimum and maximum with the medium).

Site of collection	% (Min.-Max.)	Medium
Water samples		
Site 1 point 1	75% (9/12) $6.93 \times 10^5 - 4.6 \times 10^7$	1.7×107
Site 1 point 2	58.3% (7/12) $1.2 \times 10^6 - 5.2 \times 10^7$	2.3×10^7
Site 1 point 3	66.7% (8/12) $2.9 \times 10^6 - 3.75 \times 10^7$	9.1×10^6
Site 1 point 4	50% (6/12) $2.4 \times 10^5 - 8.9 \times 10^7$	1.6×10^7
Site 2	66.7% (8/12) $1.53 \times 10^5 - 1.2 \times 10^7$	3.8×10^6
Site 3	58.3% (7/12) $8.9 \times 10^5 - 8.6 \times 10^7$	4.9×10^7
Site 4	75% (9/12) $2.2 \times 10^6 - 9.1 \times 10^7$	1.66×10^7
Sediment samples		
Site 1	58.3 % (7/12) 1.98×10^6 - 5.24×10^8	1.0×10^8
Site 2	33.3%(4/12) $7.9 \times 10^6 - 4.3 \times 10^7$	1.8×10^7
Site 3	50% (6/12) $2.3 \times 10^7 - 7.7 \times 10^8$	2.0×10^8
Site 4	50% (6/12) $3.5 \times 10^6 - 8.8 \times 10^7$	3.3×10^7

As showed in Figure 30 (a, b, and c), which presented the complete profile of HAdV as detected in water and sediment samples at each point of collection during one year (2014) where the percentage of HAdV GC in the collected water samples was: site 1 point 1 (surface water), 75% (9/12); from site 1 with different depth (0.9 m), 58.3% (7/12); from site 1 (5.5m), 66.7% (8/12); from site 1 (8 m), 50% (6/12); For sediment samples collected from the same site 1, 58.3 % (7/12); water samples collected from site 2, 66.7% (8/12) sediment samples collected in this site 2, 33.3% (4/12); water samples collected from site 3, 58.3% (7/12) sediment samples, 50% (6/12) ; water samples collected from site 4, 75% (9/12) and sediment samples, 50% (6/12).

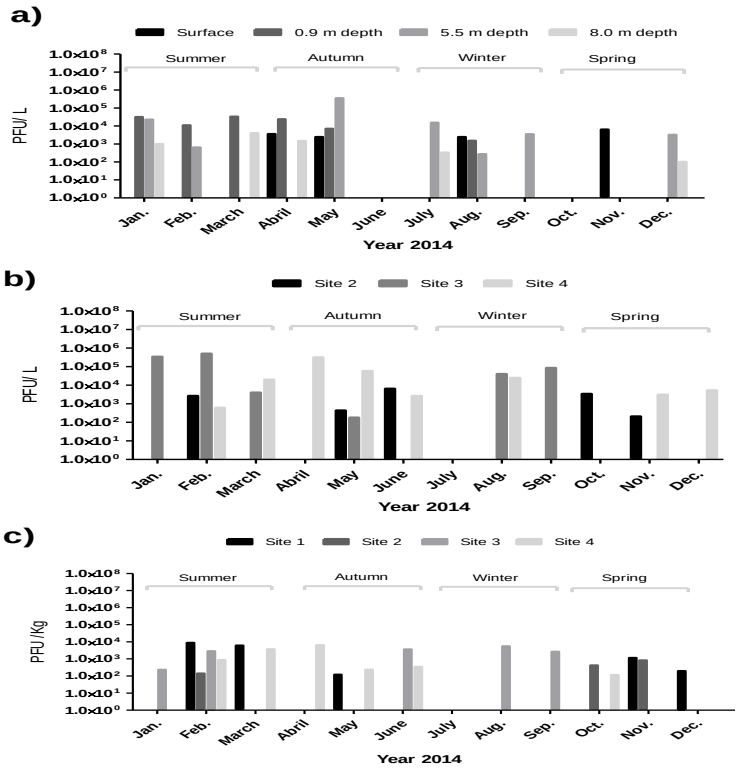
Figure 30: HAdV GC profiles during the collection of water and sediment samples. In "a", different depths of collection in site 1 (center of Peri Lagoon at surface water, 0.9 meter in deep, 5.5 m in deep and 8 m in deep); "b", water samples from sites 2, 3 and 4; "c", sediment samples from sites 1, 2, 3 and 4.



3.4 HAdV infectivity

Infectious HAdV was present in 70% (23/33) of total surface water samples, ranging from 1.8×10^2 PFU/Kg to 5.3×10^5 PFU/Kg while at different depths of water column was 86% (6/7), 87% (7/8), and 83% (5/6), respectively as shown in Fig. 31 (a, b), average number of PFU as detected by plaque assay in different depths was shown above in Table (5). Regarding to sediment samples, infectious HAdV was detected in 83% (19/23), ranging from 1.2×10^2 to 8.9×10^3 PFU/Kg as shown in Fig. 31 (c).

Figure 31: HAdV PFU profiles during one year of collection in water and sediment samples. In "a", different depths of collection in site 1 (center of Peri Lagoon at surface water [point 1], 0.9 meter in deep [point 2], 5.5 m in deep [point 3] and 8 m in deep [point 4]); "b", water samples from sites 2, 3 and 4; "c", sediment samples from sites 1, 2, 3 and 4.



3.5 Detection of RVA and HAV GC in water and sediment samples

The RVA GC were present in 33% (16/48), ranging from 1.1×10^3 to 5.4×10^5 GC/L as shown in Fig. 32 (a and b). The HAV GC were present in 27% (13/48), ranging from 1.1×10^2 to 4.3×10^3 GC /L as shown in Fig. 33. The overall averages of GC for RNA viruses (RVA, and HAV) found in the Peri Lagoon are shown in Fig. 32 (a and b) and Fig. 33, respectively. For sediment samples, RVA GC were present in 18.75% (9/48) ranging from 3.2×10^2 to 2.9×10^3 GC/Kg as shown in Fig. 32 (c). No HAV GC were detected in the sediment samples along one year of monitoring.

Figure 32: RVA GC profiles during one year of collection in water and sediment samples. In "a", different depths of collection in site 1 (center of Peri Lagoon at surface water [point 1], 0.9 meter in deep [point 2], 5.5 m in deep [point 3] and 8 m in deep [point 4]); "b", water samples from sites 2, 3 and 4; "c", sediment samples from sites 1, 2, 3 and 4.

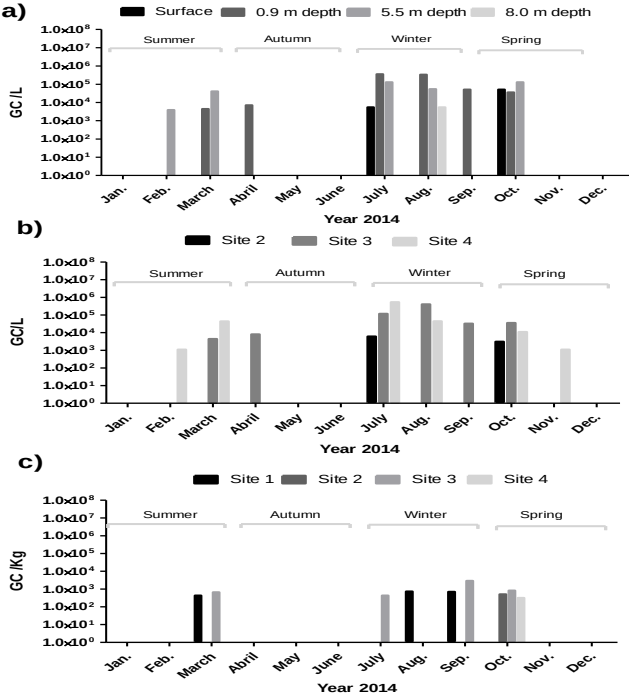
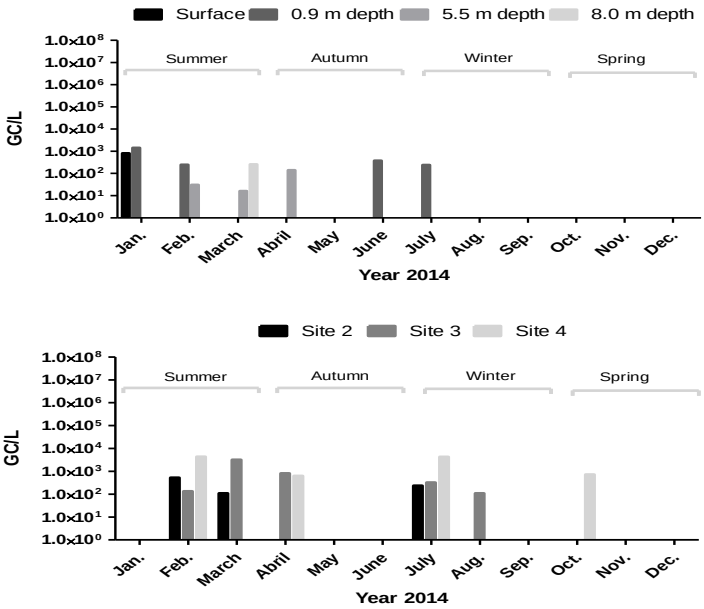


Figure 33: HAV GC profiles during one of collection in water. In "a", different depths of collection in site 1 (center of Peri Lagoon at surface water [point 1], 0.9 meter in deep [point 2], 5.5 m in deep [point 3] and 8 m in deep [point 4]); "b", water samples from sites 2, 3 and 4.



Distribution of HAdV was similar in all depths of water column with average 2.3×10^7, 9.1×10^6, and 1.6×10^7 GC/L in 0.9, 5.5, and 8.0 meters respectively. However the distribution of RVA, and HAV were more frequent in 0.9 m than in other water column depths with average 1.3×10^5, and 5.6×10^2 GC/L, respectively. Water transparency was measured by Secchi disk in each collection time over one year (Secchi depth ± 0.9m).

A positive correlation between HAdV and HAV was noted in all depths of water column. Number of detection times for each virus (HAdV, RVA, HAV, and SOMCPH) over one year surveillance at different depths of water column was completely different as shown in Fig. 34. Also average concentrations of HAdV, RVA, HAV, and somatic coliphages (SOMCPH) detected in different depths of water column are shown in Table (6).

Figure 34: Detection times for HAdV, RVA, HAV, and SOMCPH in different depths at site 1 (centre of lagoon) ($P>0.05$ $P\geq0.05$; compared by one-way ANOVA test.

HAdV (■), RVA (■), HAV (■) and SOMCPH (■),

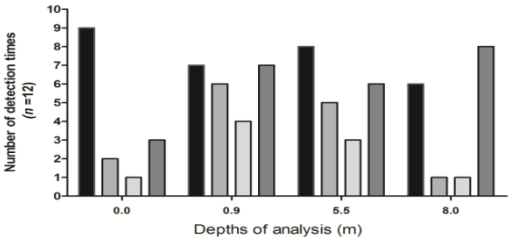


Table 6: Average concentration of HAdV, RVA, HAV, and SOMCPH detected in different depths of water column at the centre of Peri Lagoon.

Parameters	0.9 m depth Mean (Max.-Min.)	5.5 m depth Mean (Max.-Min.)	8 m depth Mean (Max.-Min.)
HAdV (gc/L)	2.3×10^{7} (5.2×10^{7}- 1.20×10^{6})	9.1×10^{6} (3.8×10^{7}- 2.9×10^{6})	1.6×10^{7} (8.7×10^{7} - 1.1×10^{6})
HAdV (PFU/L)	2.1×10^{4} (3.4×10^{4} – 1.6×10^{3})	5.8×10^{4} (3.6×10^{5} – 2.8×10^{2})	1.4×10^{3} (4.1×10^{3} - 1×10^{2})
RVA (gc/L)	1.3×10^{5} (3.6×10^{5} – 4.5×10^{3})	8.2×10^{4} (1.3×10^{5} – 3.9×10^{3})	5.5×10^{3} ND
HAV (gc/L)	5.75×10^{2} (1.4×10^{3}– 2.4×10^{2})	6.3×10^{1} (1.4×10^{2}– 1.6×10^{1})	2.6×10^{2} ND
Somatic coliphage (PFU/L)	7.2×10^{4} (2.8×10^{5}– 1.9×10^{4})	9.5×10^{4} (2.7×10^{5}– 1.3×10^{3})	1.4×10^{5} (2.8×10^{5}– 1×10^{3})

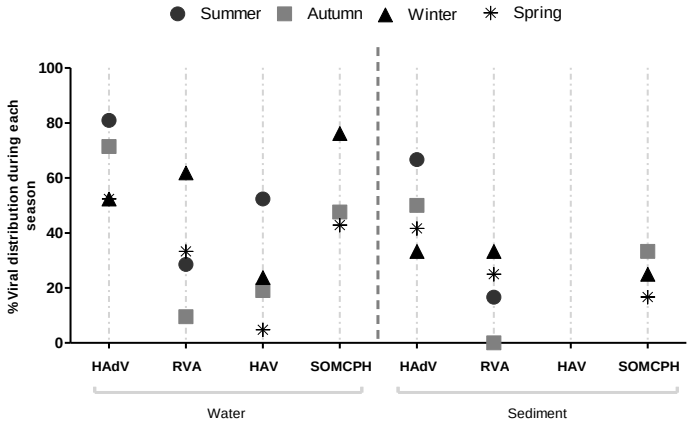
(ND = not detected)

3.6 Seasonal variation for HAdV, RVA, HAV, and SOMCPH

The overall mean percentages for the seasonal occurrence of the four viruses showed that these viruses have a seasonal trend where HAdV and HAV were more frequent in summer (81% and 52%, respectively) in water samples. Regarding to sediment samples, HAdV was also prevalent (66% for HAdV) while HAV was not detected in all sediment samples. Contrary, RVA and SOMCPH were more frequent during winter (62% and 76%, respectively) in water and (33% and 25%, respectively) in sediment, respectively. HAdV was the most prevalent

virus detected in our study. However, the seasonal distributions of these viruses were not statistically significant (Student's t-test, $P > 0.05$) as shown in Fig. 35.

Figure 35: Viral distribution (percentages) in water and sediment samples during one year of collection ($P>0.05$);



3.7 Correlation of physical-chemical parameters with presence of HAdV

Physical-chemical data (temperature, pH, DO and cond.) for all point of collection and nutrients (nitrite, nitrate, phosphorus, ammonia, nitrogen, and total phosphate) for site 1 of collections of Peri Lagoon, were submitted to the t-test , two-way ANOVA and correlation Pearson test with the HAdV. By applying t-test we found that there are no correlation between any of physical-chemical parameters with the presence of HAdV GC ($P>0.05$) (Pearson test-ANOVA) (r ranged from 0.01 to 0.3).

3.8 Correlation of SOMCPH with presence of HAdV

Presence of SOMCPH and HAdV in either water or sediment samples for all sites was not statistically significant ($P>0.05$) by using Tow way-ANOVA test. Also by applying Pearson correlation test for these results we found that, there is no positive correlation between the presence of SOMCPH and HAdV (either for genome or infectious units) ($r = -0.23$).

4. DISCUSSION OF CHAPTERS I and II

Waterborne viral infections are among important causes of human morbidity, and related diseases continue to pose public health threat and socioeconomic implications worldwide. According to existing reports in the literature, there are hundreds of different types of human viruses present in human sewage, which can become a source of contamination of drinking and recreational water. In many countries, water quality is evaluated according to bacteriological standards, even though bacterial contamination is not correlated with the presence of human enteric viruses and other pathogens (FONG et al., 2005).

Most of waterborne diseases are due to unsafe water supply and inadequate sanitation. Drinking and recreational water are the main ways of human viral contamination, which can be responsible for multiples diseases. Water-related human pathogenic viruses include HAdV, HNoV, rotaviruses, astroviruses, HAV and HEV (BOFILL-MAS et al., 2013). Drinking and recreational waters, such as swimming pools and seawater are also contaminated and outbreaks of HAdV infection are frequently described in day care centers, hospitals and other public places (MENA; GERBA, 2008).

Coastal lagoons are important environments for water suppliers and sanitation processes and are considered an important contributor to underground water. Contamination of lagoons with enteric viruses due to the illegal discharge of domestic sewage represents a significant risk to public health (MORESCO et al., 2012; ASLAN et al., 2011). In this study, we investigated the presence of human viral pollution (in the form of HAdV, RVA and HAV) in surface water and sediment over two year period (2013, 2014) in order to investigate the role of sediments as reservoirs for these pathogens and also to see the possible influence of seasonality in the presence of these viruses.

Real-time PCR was applied to quantify the presence of HAdV. HAdV proved to be the most prevalent virus in this study during the two years of collection, both in surface water and sediment samples with an indicated peak in summer collections.

These results corroborated with other studies, which found that HAdV is the most prevalent virus in different environmental matrices; HAdV was detected in 75% of lagoon water samples tested by RIGOTTO et al., (2010) using nested PCR. Additionally, other studies have reported the high incidence of HAdV in different aquatic environments (HUNDESA et al., 2006; CHOO; KIM, 2006; MIAGOSTOVICH et al., 2008; MORESCO et al., 2012; FONGARO et al., 2012 and 2013). The lower number of HAdV positive sediment

samples may be due to some obstacles in the method for viral elution in these matrices. These methods can interfere with the enzymatic amplification of the nucleic acids in these samples due the presence of inhibitors (such as humic acids, proteins, organic and inorganic compounds) that inhibit amplification of the viral genome target (SANO et al., 2003; ALBINANA-GIMENEZ et al., 2009; SIDHU; TOZE, 2009; ROCK et al., 2010).

However, for the positive sediment samples, the number of HAdV particles (GC) was two or three logs higher than in water samples (chapter I), while the number of HAdV particles (GC) was one or two logs higher than in water samples (chapter II). However this difference in logs may be due to that Sangradouro River has an illegal dumping of sewage, from surrounded population. HAdV tends to adsorb onto the suspended particles due to its high sorption capacity and its size, which has an effect on the interaction processes with the environmental colloidal particles (DOWD et al., 1998; WONG et al., 2013).

Detection of infectious viruses in environmental samples requires the use of adequate susceptible cell lines in which the viruses can propagate and produce cytopathic effects (CPE) (RODRÍGUEZ et al., 2009). As the presence of the viral genome does not provide information on infectivity, a viability assay was performed to detect the infectious HAdV particles in the samples containing HAdV nucleic acid.

In Sangradouro River (chapter I), the majority of HAdV positive samples from the summer collection did contain infectious particles, but any infectious units were detected neither in the surface water nor in sediment samples from the winter collection from the collection of the first year (2013). The degradation of the viral particles may be due to various factors, such as pH or U.V. light incidence, in addition to other environmental components, such as the presence of proteases, (such as RNAses, and DNAses), which can influence the resistance and inactivation of viruses in aquatic environments by causing conformational changes in the viral capsid (WARD et al., 1986; BATTIGELLI et al., 1993; CARTER, 2005, FONG; LIPP, 2005, CHOI; JIANG, 2005, FONGARO et al., 2013). The infectivity test is important when quantitative microbial risk assessment is the goal. To assess possible damages to the HAdV capsid, which results in a lack of protection for the nucleic acids, the DNAse pre-treatment of samples prior to nucleic acid extraction was applied to all samples analyzed in the present work. Previous studies have reported this enzymatic technique as a simple alternative technique to give inferences about

potential viral infectivity without using laborious cell culture techniques (RODRÍGUEZ et al., 2009; VIANCELLI et al., 2012; FONGARO et al., 2013).

This assay allows for differentiation of undamaged viruses (with protected genomes) from damaged viruses (with unprotected genomes) or even free viral genomes. This assay nevertheless is unable to differentiate inactivated virus with genetic material that is still protected by viral capsid (RODRÍGUEZ et al., 2009; FONGARO et al., 2013). This limitation was proved in this work, because not all samples containing undamaged HAdV as detected by qPCR were infectious by plaque assay. The notable differences among these results highlight the critical importance of combining, whenever possible, molecular and cell culture techniques during the monitoring of viruses in the aquatic environment.

In Peri Lagoon (chapter II) HAdV was found to be more prevalent in water and sediment samples (64%, and 48%, respectively) than other enteric viruses detected by qPCR in different aquatic matrices (water and sediment). High diversity of HAdV in water samples was documented recently by OGORZALY et al., (2015) by using a Next-Generation Amplicon Sequencing (NGS) Approach. This virus proved to be prevalent in the water samples collected during the first one year of monitoring with an indicated peak in summer collections which coincides with summer holiday period and a high increasement of the fluctuant population. These results corroborated with other studies from our and other groups (GENTHE et al., 1995; WONG et al., 2009; RIGOTTO et al., 2010; MORESCO et al., 2012; FONGARO et al., 2012; GARCIA et al., 2012). The viral load in water samples ranged from 10^5 GC/L to 10^7 GC/L. The detection frequency found in the present study was nearly equal in comparison to previous studies related to surface water: done by FONGARO et al., (2012) (10^6 to 10^8 GC/L), and by GARCIA et al., (2012) (average of 10^7 GC/L). However, other studies reported viral loads lower in 2 or 3 logs from those presented here in surface water: YE et al., (2012) (10^2–10^4 GC/L), CHOI and JIANG (2005) (10^2–10^4 GC/L), HARAMOTO et al., (2010) (10^3–10^5 GC/L), JURZIK et al., (2010) (10^3–10^5 GC/L).

In Peri Lagoon (chapter II), a higher positivity of infectious HAdV was noted in sediment samples than in water samples (83% and 76%, respectively). The viral load for HAdV was almost the same in both matrices (water and sediment) ranging from 10^2 to 10^4 PFU/L and 10^2 to 10^3 PFU/Kg, respectively. HAdV load detected by STAGGEMEIER et al., (2015) was 3 times higher in sediment samples than in water and this

difference may be due to the detection technique used (ICC-RT-qPCR) which is more sensitive than plaque assay and able to detect HAdV as low as 1×10^2 genome copies per milliliter of infectious viral particles in the environmental water samples according to FONGARO et al., (2013) and this reinforce the idea about the combining of molecular and cell culture technique during the monitoring of viruses in the aquatic environment to maintain a reliable result for public health.

HAdV have been indicated as potential marker of viral contamination in water (CALGUA et al., 2008; BOSCH et al., 2011; WYN-JONES et al., 2011; WYER et al., 2012) since this virus has DNA genome, which confers more resistance to environmental stressors, such as UV radiation, temperature, chlorine concentration and pH variation, including sewage treatment procedures (LECHEVALLIER; AU, 2004; FONG; LIPP, 2005), moreover, HAdV were found to be more prevalent than other enteric viruses (WONG et al., 2009; WYN-JONES et al., 2011; GARCIA et al., 2012, FONGARO et al., 2012; MORESCO et al., 2012).

On the other hand, there are several candidate viral indicators to predict virus contamination in environmental waters. Several studies indicate that bacteriophages could serve as viral indicator for estimating enteric viruses in water (HAVELAAR, 1993). Three groups of bacteriophages have been proposed as potential viral indicators for estimating sewage contamination and human enteric viruses in water based on their similarities to human enteric viruses in morphology, nucleic acid composition, sources and occurrence in contaminated water: F-specific RNA coliphage, phages of *Bacteroides fragilis*, and somatic coliphages.

In chapter II, we investigated the presence of somatic coliphages in water and sediments over one year monitoring, to see the possible influence of seasonality in the presence of these viruses and also to show a possible correlation between them (somatic coliphage and DNA & RNA viruses in this study).

In our study (chapter II), somatic coliphage proved to be prevalent during the winter and spring seasons along one year of collection and it was completely absent during the summer season. This may be due to the concentration of environmental phage populations. Several authors have unsuccessfully tried to isolate phages through direct methods, and others, although occasionally successful, but did not publish their findings (ACKERMANN, 1997).

All of the positive samples for somatic coliphages by double agar layer method showed to be positive for the presence of *E. coli.* However there is no positive correlation was found between human adenoviruses quantified by real-time PCR or by plaque assay and culturable coliphages, indicating that the detected adenoviruses might be originated by human feces and respiratory secretions while coliphages might be generated by other natural sources such as birds, animal feces, and/or regrowth in nutrient-rich soil (DESMARAIS et al., 2002). Our data confirm findings from other studies made in different regions of the world; JIANG et al., (2001) reported the presence of HAdV (by nested PCR) and culturable coliphages in coastal waters of Southern California in four site of collection and they noted that the presence of human adenovirus was not significantly correlated with the concentration of coliphage; GRIFFIN et al., (1999), detected human enteric virus (polioviruses, coxsackie A and B viruses, echoviruses, hepatitis A viruses, Norwalk viruses, and small round-structured viruses) in 95% of samples collected from Florida coastal water by PCR whereas a few or no coliphage was detected in their samples; while CHUNG et al., (1998) reported a significant association of F-specific phage with human enteric viruses in oysters and their harvest waters. By using real time PCR, CHOI; JIANG (2005) made a study on two urban rivers in California and JURZIK et al., (2010) made a study on river Ruher, Germany and they found no significant correlations between HAdV and culturable coliphages.

The RVA was present in 33% of water and 19% of sediment samples collected in Peri Lagoon and 33.2% of water and 10.4% of sediment samples collected in Sangradouro River, suggesting a cross contamination with human sewage in both environments. Rotavirus is the most common cause of severe diarrheal diseases in infants and young children worldwide causing > 600,000 deaths each year under 5 years of age and can be shedding in high concentration in the feces of infected persons ($>10^{10}$ virus particle/g) with a small infectious dose <100 virus particles. Many studies demonstrated a high prevalence of RV in different matrices as following, in the sewage: MELEG et al., (2008) (91%); RODRIGUEZ-DIAZ et al., (2009) (67%), FUMIAN et al., (2010 and 2011) (47%, 100%); in the river contaminated with sewage: RODRIGUEZ-DIAZ et al., (2009)(83%); in stream waters: MIAGOSTOVICH et al., (2008) (44%); in the surface water of river and lagoon: HAMZA et al., (2009) (90%), JURZIK et al., 2010 (64%), FONGARO et al., (2012) (65%),and in fluffy sediment: RAO et al., (1986) (40%).

However, the low incidence of these RNA viruses in sediment samples may be due to their weak absorption on sediment particles or due to their quick inactivation in sediments. Previous studies that reported the incidence of rotavirus in sediments done by CHUNG; SOBSEY (1993) and GREEN; LEWIS (1999) also demonstrated a low incidence (12.5%) and suggested that the viral adsorption to sediment may inactivate this virus. However, these authors reported a high incidence of HAV in sediment samples contaminated by wastewater (87.5%). Other studies regarding HAV incidence in Brazil have also shown a low incidence in environmental samples (RIGOTTO et al., 2010).

In Brazil, attenuated G1P[8] vaccine, Rotarix (GlaxoSmithKline, Rixensart, Belgium), was included in the Immunization Program since 2006 (FUMIAN et al., 2011). However a recent study done by GUERRA et al., (2015) noted that G2P[4] was the predominant RV strain circulating in Belém, Brazil and detected in 57.2% of circulating strains over their whole study period (three years) which suggests a shift in genotype distribution since 2006 (CARVALHO-COSTA et al., 2009, GUERRA et al., 2015). These data make the continuous RV genotyping monitoring is needed for discovering others shifting.

Other reports demonstrated a low incidence of HAV in different environmental samples in lagoon water: RIGOTTO et al., (2010) (17%), FONGARO et al., (2012) (12%); in sewage: FORMIGA-CRUZ et al., (2005) (20%); in raw and treated sewage in Brazil: VILLAR et al., (2007) (32%). However in one study done at the Buffalo River, South Africa by CHIGOR; OKON (2012), HAV was the most prevalent detected viruses with 43% of positivity. Our results suggested that there is a possible contamination route via human sewage for Peri Lagoon (the present studied area) or even this contamination can be due to recreational activities such as bathing in these areas during vacation period. Moreover, no samples have been found positive for HAV in sediments.

The detection and quantification of enteric viruses in matrices reported in these chapters support the idea related to solid-associated viruses and its importance to take in consideration during a routine monitoring of the aquatic environment to reduce the risk of illness from enteric viruses. Many authors in the literature found that viral abundance in sediment exceeds that in the water column by an order of magnitude (DANOVARO et al., 2002; DANOVARO; MIDDELBOE, 2010). Also Statistical analysis revealed no significant correlations between the

physicochemical parameters with the presence of these viruses and these finding were confirmed by other reports in the literature (LEE et al., 2013; VECCHIA et al., 2015).

The high prevalence of HAdV in the surface water and sediment samples along two years of monitoring indicates that there was no seasonal difference. So, the prevalence of HAdVs is ubiquitous in the environment all year round, a result that has been confirmed by other studies done by FORMIGA-CRUZ et al., (2005) and RIGOTTO et al., (2010). This high incidence may be due to the high stability of HAdV in the aquatic environment and its sporadic shedding in the faeces of most adults (WYN-JONES et al., 2009). Regarding somatic coliphage we noted a higher incidence during winter months along our study in the second year of collections.

RVA showed a marked winter seasonal peak in water (45.5% in [chapter I], 62% [chapter II]) as well as sediment samples (12.5% [chapter I], 33% [chapter II]), which has also been reported in previous studies (COOK et al., 1990; LEVY et al., 2009; PAYNE et al., 2011, CHIGOR; OKOH, 2012). Some authors had demonstrated higher levels of RVA during the cold, drier months (STEELE; GLASS, 2011), while others suggested that this seasonal pattern may vary according to the RVA serotype (SARKAR et al., 2008; JAGAI et al., 2012).

However, the seasonal prevalence of these viruses was not statistically significant. In the present study, HAV displayed a higher incidence during the summer (45.8% [chapter I], 52% [chapter II]). However, CHIGOR; OKOH, (2012) reported higher detection of HAV in South Africa during the winter and spring seasons (43%). Another study performed in Brazil by RIGOTTO et al., (2010) documented a seasonal pattern for HAV during the spring and winter seasons, with a high incidence during the months of October and July. Another study done in Rio de Janeiro by VILLAR et al., (2002) reported a peak of HAV incidence during hot and rainy months. However, we must take into consideration the fact that many cases are underreported and others are asymptomatic.

Distribution of aquatic microbial organisms in the water column can be varied depending of varies factors such as: their velocity of settling and wind, suspended solids in water column, presence of anaerobic or aerobic predators and also for the intensity of natural sunlight through the water column. HAdV was detected in the present study in the surface water and in all depths of water column (0.9m, 5.5m, and 8m), this may be due to nucleic acid nature of this virus that increase its resistance to various environmental factors such as UV radiation and

temperature which are known to cause conformational changes in the viral capsid (SIRIKANCHANA et al., 2008; EISCHEID et al., 2009). While RVA, HAV, and somatic coliphage were shown to have a high increase with depth from the surface towards while they have a slightly presence in the surface water of the centre of Peri lagoon. Therefore, the lagoon centre deep offers stable environments for some microbial life throughout water layers. While the other three collections sites in the present study we could see this difference because of maximum deep is around 1.5m. The vertical patterns observed in this study for the detected enteric viruses imply that throughout the depths of water column the loss of these pathogens should be low because of the darkness and lower temperature (MURRAY; JACKSON 1993; HARA et al., 1996; COLOMBET et al., 2006). However, there is no information available in the literature about the presence and distributions of enteric viruses throughout different depths of water column. But these results forced us to make a prospective study using different microcosm seeded artificially by different enteric viruses in different realistic exposure conditions for natural sunlight and dark condition.

5. CONCLUSIONS

-The results showed that in the Island of Florianopolis, recreational waters and lagoons, which are the main resources for drinking water, are being contaminated by enteric viruses, either by sewage runoff or by its streams and interconnections.

-This study highlighted the importance of HAdV as viral indicator parameter in addition to bacteriological parameters in the monitoring of recreational water microbiologically. Also RV, HAV, HAdV and SOMCPH should be indexed for human fecal pollution in estuarine sediments as the only refuge of solid associated viruses,

- SOMCPH in our study, did not shown a good performance as indicator for the presence of viral contamination in water samples,

-The results highlighted the importance of evaluating the ecosystem interconnections of Sangradouro River in order to enhance the safety of water as a drinking and recreational resource in this environment. This water may otherwise pose a threat to the population that is mainly responsible for the degradation of its quality in this lagoon and neighborhood.

-The HAdV integrity test by enzymatic treatment (DNAse I) is a less laborious method than cell culture technique and proved to be feasible to detect potentially infectious viral particles;

-The sediments also proved to act as shelter for enteric viruses, increasing their concentrations and being responsible for the return of these pathogens to the water column when resuspended.

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CHAPTER III

STABILITY OF HUMAN ADENOVIRUS AND MURINE NOROVIRUS IN WATER AND SEDIMENT SAMPLES USING LABORATORY MICROCOSM SCALE

CHAPTER III

1. INTRODUCTION

This chapter will present and discuss the results of HAdV and MNV-1 stability in laboratory scale microcosms kept under two conditions: natural condition receiving natural sun light and full dark condition. To prepare the microcosms we used surface water and sediment samples catched at Peri Lagoon.

2. MATERIAL AND METHODS

2.1 Collection of samples

Surface water and sediment samples were collected at the center of Peri Lagoon (site 1 as described in Chapter II), Florianopolis, Santa Catarina State, Brazil (July, 2014).

2.2 Physical-chemical analysis

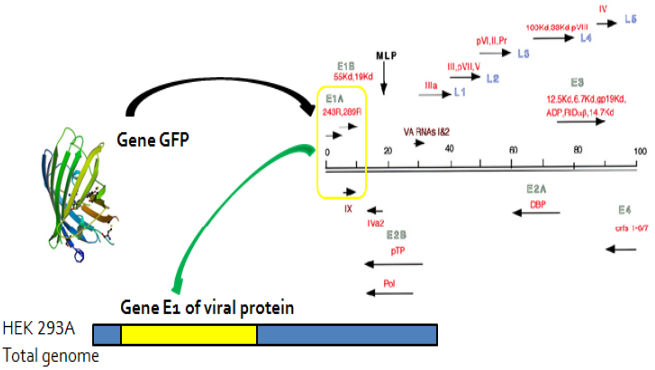
Water temperature (WT), conductivity (Cond), pH, and dissolved oxygen (DO) were measured *in situ* with specific probes (WTW-Multi350i) for all of samples after collection, as outlined in the standard methods for the examination of water and wastewater described by the American Public Health Association (APHA, 1998). Water samples were taken to the laboratory to quantify Ortho-Phosphate (PO_4), Total Phosphate (TP), Nitrite (NO_2.N), nitrate (NO_3.N), ammonium nitrogen (NH_4.N), and total Nitrogen (TN) were determined in filtered water samples using a Millipore AP40–47 mm glass fiber (GOLTERMAN et al., 1978; KOROLEFF, 1976) (these analyzes were carried out in partnership with the Laboratory of Continental Water Ecology, Department of Ecology and Zoology at the Federal University of Santa Catarina, under the coordination of Prof. Dr. Mauricio Mello Petrucio).

2.3 Recombinant Adenovirus as a model for DNA enteric viruses

Recombinant human adenovirus (rHAdV) which was used in this chapter is related to serotype 5 but has some modification in its genome region. E1 gene (E1A and E1B) was removed and replaced by the gene of green fluorescent protein (GFP). HEK 293A (cell line of human embryonic kidney) was genetically transformed to express the viral protein E1 as shown in Fig. 36, thereby this cell line is susceptible and permissive to rAdV. Therefore when rAdV replicate, GFP is transcribed along with the virus genome emitting a green fluorescence that can be easily detected by fluorescence microscope (DAN et al., 2010;

WEAVER; KADAN, 2000). By using this model of recombinant adenovirus, there is no need for further immunologic methods making this model more rapid, inexpensive, and more specific (LI, HE, JIANG, 2010). However in the literature there are few studies related to the stability and infectivity of HAdV in the aquatic environment using rHAdV. (GARCIA et al., 2015; NASCIMENTO, et al., 2015).

Figure 36: Schematic structure of human recombinant adenoviruses (rHAdV) related to serotype 5, composed by the green fluorescence protein (GFP) gene incorporated and genetically transformation of HEK293A to express the viral E1 protein



Source: Author

2.4 Cell lines and preparation of viral stock of rHAdV, and MNV-1 for seeding experiment in water and sediment samples

The cell lines HEK 293 (human embryonic kidney) and RAW 264-7 (*Mus musculus*, mouse macrophages), permissive to rHAdV and MNV-1, respectively were used for the experiments. Both were cultivated in sterile cell culture flasks (75cm^2), with Dulbecco's Minimum Essential Medium Eagle`s salts (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% HEPES, until they reached confluence (24 h, 37 ºC, and 5% CO_2). Then the growth media was removed and 1.0 mL for each viral suspension was inoculated separately in each cell culture flask. After 1h for viral adsorption at 37°C, 20 mL of cell maintenance media were added consisting of the same growth medium, but with 2% FBS. Cell cultures were always monitored and compared with an uninfected control cell for checking the appearance of

cytopathic effect (CPE). In case of rHAdV, the cells were monitored every day, and observed using an inverted fluorescence microscope which allowed the visualization of cells with green fluorescence (GFP) due to transcription and translation of GFP protein, which means viral replication. In case of MNV-1, the cells were observed under inverted microscope until reach 100% of cytopathic effect (CPE), characterized by rounded cells and even detachment from the surface, destroying the cell monolayer.

The cell culture flasks that contain infected cells with rHAdV or MNV-1 were then frozen at -80 °C and thawed at 25°C three times for cell lysis. Then, the virus suspension was transferred, separately to a tube and centrifuged at 3,500 χg for 4 min. at 4°C to remove the cells debris and separate the supernatant. The supernatants were then used to infect other flasks with the appropriated cells in order to expand the amount of virus stocks to be used for the further experiments.

2.5 Cytotoxicity assay

The cytotoxicity of surface water and sediment samples were evaluated either on HEK 293 or in RAW 264-7 cells. These cells were propagated in sterile 24-well plates at a density of 2.5×10^5 and 1×10^6 cells per well, respectively. Surface water and sediment samples were pretreated with 1% antibiotic/antifungal (100 U penicillin G / mL / streptomycin 100 µg /mL amphotericin B 0.25 µg /mL) and diluted in 1X DMEM in the following proportions: 1:1, 1:2, 1:4, 1:8. An inoculum of 100 µL of each dilution in triplicate was placed in contact with each type of tested cells for 1h at 37 °C under 5% CO_2 atmosphere and uniform mixing every 15 min. Subsequently, the inoculum was removed and 1 mL of DMEM containing 1% PSA was added to the cells, two cell controls were maintained for each plate and these cells contained only medium. The plates were incubated at 37°C in 5% CO_2 atmosphere and the cells monolayer was monitored for 3 days in case of RAW 264-7 cells and only one day in case of HEK 293.

Cells containing inoculated test samples were compared with control cell under a light microscope. Sequentially, cells were fixed and stained with 300µL of Naphthalene Black solution (0.1% Naphthalene Black, 5% Acetic Acid, pH 2.3-2.4). The cell monolayer was stained and examined for the non-cytotoxic dilution, for each of tested samples in order to be further used for infectivity assays.

2.6 Monitoring the infectivity of rHAdV in surface water and sediment samples by Fluorescence microscopy (FM)

These cells were previously cultivated in 48-well tissue culture plates at a density of 2.5×10^5 cells / well, with Dulbecco's Minimum Essential Medium Eagle`s salts (DMEM) (Sigma) supplemented with 10% FBS and 1% HEPES, until they reached confluence and were incubated at 37°C under 5% CO_2 for 24 h. Then 100 µL viral fluids as a positive control or sample were tested using non-cytotoxic dilutions. The samples were serially diluted (10 fold) previously as following: for viral fluid 50 µL viruses were diluted in 450 µL of DMEM 1X until 10^{-5}. Regarding the samples, 50 µL were diluted with 450 µL of 1X DMEM with 1% PSA and serially diluted until 10^{-5}. In the cell control wells (negative control) was added only DMEM 1X. After 1h at 37°C under 5% CO_2, 0.4 mL of maintenance medium was added per well (1X DMEM, 2% FBS, 1% HEPES and 1% PSA) to 48 plate wells. After 24 h the cell supernatant was aspirated and the plate was visualized directly under inverted fluorescence microscope. The number of fluorescent cells in the highest possible dilution was counted and the viral titer expressed in focus forming units (FFU/mL) according to the following formula:

$$\frac{\text{N. of fluorescence cell (average of triplicate) x Reciprocal dilution}}{\text{Volume of inoculum in each well (mL)}}$$

The detection limit of the technique was established according to the highest dilution where it was possible to count the infected cells.

2.7 Study of the infectivity of MNV-1 in surface water and sediment samples by plaque assay

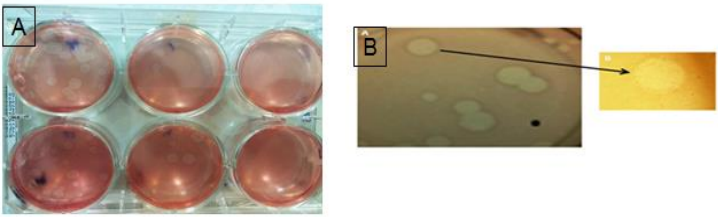
These cells were previously cultivated in 6-well tissue culture plates at a density of 1×10^6 cells / well, with Dulbecco's Minimum Essential Medium Eagle`s salts (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% HEPES, until they reached confluence and were incubated at 37°C under 5% CO_2 for 24 h. The plaque assay for the MNV-1 was based on the protocol described by BAE; SCHWAB, (2008). Briefly, 400 µL of viral fluid or sample in non-cytotoxic dilution were inoculated and incubated for viral adsorption as described above. The inoculum was removed and added the cell culture maintenance (2x MEM), diluted in melted agarose 3% (SeaPlaque Agarose low melting point, Lonza, CA, USA) in order to prevent the virus from spreading,

limiting the infection only to neighboring cells that were primarily infected. Cells were incubated for 48h at 37°C. After this period, 1.0 mL of a 0.1% solution of neutral red dye was overlaid on the agarose layer, which enabled the visualization of lysis plaques and its count after 3h as shown in Fig. 37 (a, b). The number of plaque units in the highest possible dilution was counted and the viral titer expressed in plaque forming units (PFU/mL) defined by the following formula:

$$\frac{\text{N. of plaques (average of duplicate) x Reciprocal dilution}}{\text{Volume of inoculum in each well (mL)}}$$

The technique detection limit was established according to the highest dilution where it can perform plate count.

Figure 37: Illustrative picture of Plaque assay for MNV-1 (A), aspect of the plaque forming unit (B).

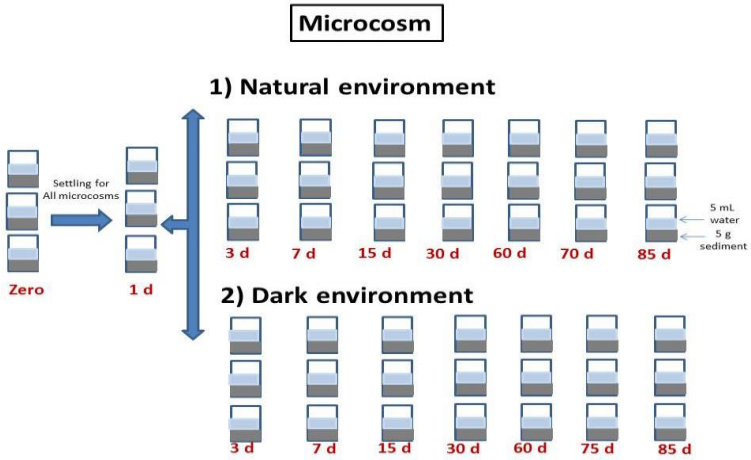


Source: Author

2.8 Microcosm design and sampling

Experiments were set-up in microcosms in a glass container (NADER-Brazil, 50 mL) filled with a sediment layer of 5 cm and an overlying water column of 5 cm as shown in Fig. 38. The microcosms were stored in two conditions: i) Dark environment (24 h dark); ii) Natural environment (+/-12 h of natural sunlight/+/-12 h of dark moon light). The microcosms were kept until 85 days starting at 19, September 2014 and finishing at 13, December 2014. Microcosms were sampled in triplicates at period's intervals for up to 85 days as shown in Fig. 38. Also triplicate samples were taken for each microcosm. Infectivity studies were undertaken with rAdV, and MNV-1 and the tests were revealed by fluorescence microscopy and plaque assay, respectively.

Figure 38: Flow chart showing the basic experimental design for microcosms establishment



2.9 Inoculation of microcosms

Stock viral suspensions were prepared as mentioned before either for rHADV (2×10^7 FFU/mL) or MNV-1 (5×10^6 PFU/mL). One milliliter from each viral stock was inoculated in each microcosm jointly and mixed during 5 min for homogenization and 1h for proper settling. Then half numbers of the microcosms were placed in dark condition (24 h) and the other half were placed in natural condition (+/-12 h of natural sunlight/+/-12 h of dark moon light). Three microcosms were used to determine the initial titer of inoculated viruses before start the experiment, 1 h after inoculation.

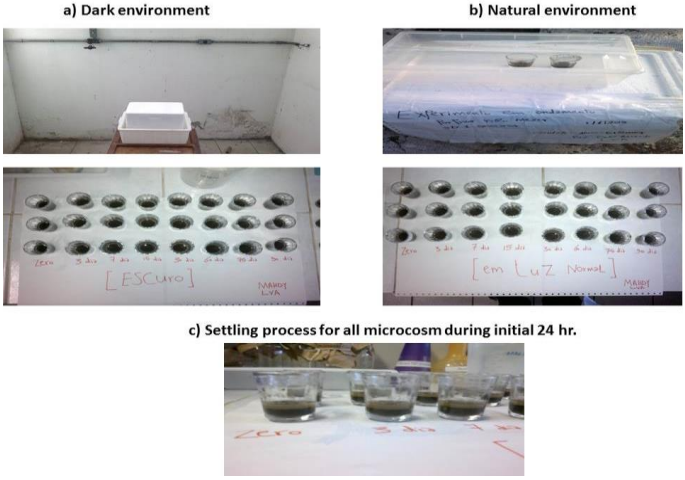
Both sediment and water from the microcosm were analyzed on days 0 (1 h after initial inoculation), 1, 3, 7, 15, 30, 60, 70 and 85 days. Three microcosms were removed per time point with triplicate samples assayed from each as shown in Fig. 39.

For each analysis, overlying water was removed and going directly for analysis with non-cytotoxic dilution. While sediment samples were obtained by first removing the remaining overlying water and going for elution step as already described. Temperature was taken for each collection sample during the experiments.

The decay rate constant (***k***), that means, the decrease in viral concentration over time, was calculated as the slope of the line when $\log_{10}$ ($\mathbf{N_t}$ / $\mathbf{N_0}$) was regressed against time, where $\mathbf{N_t}$ is the number of each virus at time **t** and $\mathbf{N_0}$ is the number of virus at time 24 h The decay

rate constant value (***k***) could then be used to calculate $\mathbf{T_{90}}$ value, which are the times required for a 1 $\log_{10}$ reduction in viral concentration.

Figure 39: Laboratory scale microcosm setup for 85 days, using a dark environment (a), natural environment (b), and the settling process during the first 24 h (c). Showing the replicate for each collection time



Source: Author

2.10 Direct elution of viruses from sediment samples

After removal of water samples from each microcosm, the sediment was gently diluted in 20 mL of modified Eagle's minimal essential medium (E-MEM) (pH=11.5) as described by STAGGEMEIER et al., (2014). The solution was homogenized by vigorous agitation (vortex) for 1 h and then centrifuged at 2.000 × g for 10 min. The supernatant was then used for further investigation. In case of water samples, they were used directly without any concentration or elution methods. Also all water and sediment samples that were collected from Peri Lagoon were concentrated as described in Chapter I to assess the presence or absence of HAdV genome copies by qPCR before starting the set-up of the microcosms.

2.11 Statistical analyses and modelling

All analyses were undertaken in triplicate and expressed as the mean ± standard deviation. Slopes of the lines from linear regression (The time for a decimal reduction of viruses, T_{90}) were calculated considering linear regression curves, using Microsoft Excel. Statistical two-way

analysis of variance (ANOVA) was employed to evaluate differences between viruses concentrations in the overlaying water compared with the sediment layer for microcosm experiments. The Pearson correlation was applied to evaluate positive or negative correlations between viral infectivity and light or dark condition (GraphPad Prism 5.0). The critical *P*-value for the test was set at <0.05.

3. RESULTS

3.1 Water characteristics and level of organic material in sediment samples

The mean values of the physical-chemical parameters measured for surface water samples from the center of Peri Lagoon are presented in Table 7. For the sediment samples, it contains granule (0.7%), sand (1.42%), silt (2.44%), and organic matter (33.5%).

Table 7: Physical-chemical parameters for surface water samples used in a laboratory scale microcosm

Parameters	Value
Temp. of water (ºC)	25
pH	7.3
Cond. (µs cm^{-1})	75.4
DO (mg L^{-1})	8.0
OP (µg L^{-1})	2.3
TP(µg L^{-1})	9.98
NO_2^-.N (µg L^{-1})	0.08
NO_3^-.N (µg L^{-1})	5.5
NH_4^+.N (µg L^{-1})	1.94

3.2 Titration of rHAdV, MNV-1 and detection limit

Stock suspension of rHAdV was titrated by fluorescence microscope at 1×10^7 FFU/L, and the detection limit was at dilution 10^{-6},

corresponding to 8.5×10^{1} FFU/mL. While stock suspension of MNV-1 was titrated by plaque assay method at 5×10^{6} PFU/L. and the detection limit was at dilution 10^{-5}, corresponding to 2.0×10^{1} PFU/mL.

3.3 Cytotoxicity assay for water and sediment sample using RAW 264-7 and HEK 293

This assay was performed to assess the non-cytotoxic lowest dilution for all samples types used in microcosm. Water and sediment samples (after elution method as described above) were diluted serially and inoculated on cell lines. Dilution 1:4 either for water or sediment samples showed no morphological changes in cell lines when compared to the control cell (only cell and medium). Thus this dilution (1:4) was the minimum dilution which was used in all samples inoculated in cell lines during microcosm experiments.

3.4 Infectivity reductions in human viruses

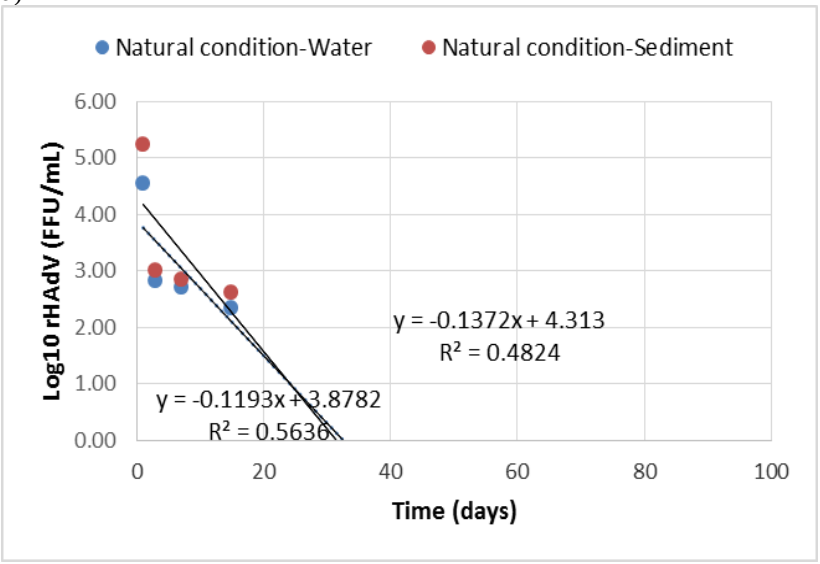
3.4.1 Recombinant Human Adenovirus (rHAdV)

The time course of decay in natural system for rHAdV are shown in Fig. 40 (a), where, as expected, effect of temperature and natural UV light on viral stabilities was observed. Temperature index along the laboratory scale microcosm was measured as shown in Fig. 41. After 15 days, rHAdV lost its infectivity in about 2.2 $\log_{10}$ (2.13×10^{2} FFU/mL) in overlaying water, about 2.6 $\log_{10}$ (4×10^{2} FFU/mL) in sediment, after 30 days we could not detect any fluorescent cells in both matrices (water and sediment).

In dark system, for sediment, rHAdV decay rates were lower and decreased along the time course. We could detect infected fluorescent cells until 70 days with a log reduction of about 2.8 $\log_{10}$ (2.7×10^{2} FFU/mL) for sediment. While in overlaying water column, the viral reduction was 2.5 $\log s_{10}$ (1.2×10^{2} FFU/mL) after 60 days; after 70 days we could not detect any fluorescent cells (below of detection limit). The $\log_{10}$ reduction values were taken based on virus concentration detected after 24 h of settling process for virus after inoculation as shown in Fig. 40 (b).

Figure 40: Survival and $\log_{10}$ reduction of rHAdV in natural environment (a) and full dark environment (b).

a)



b)

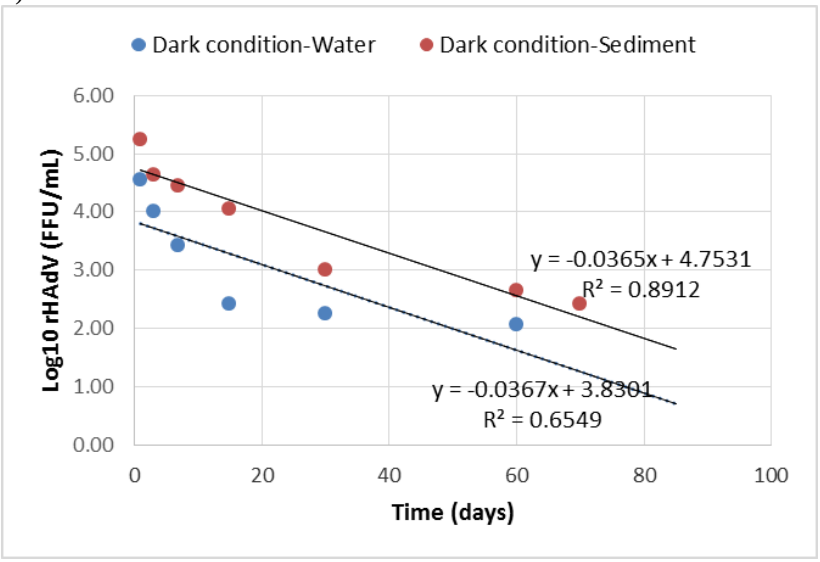
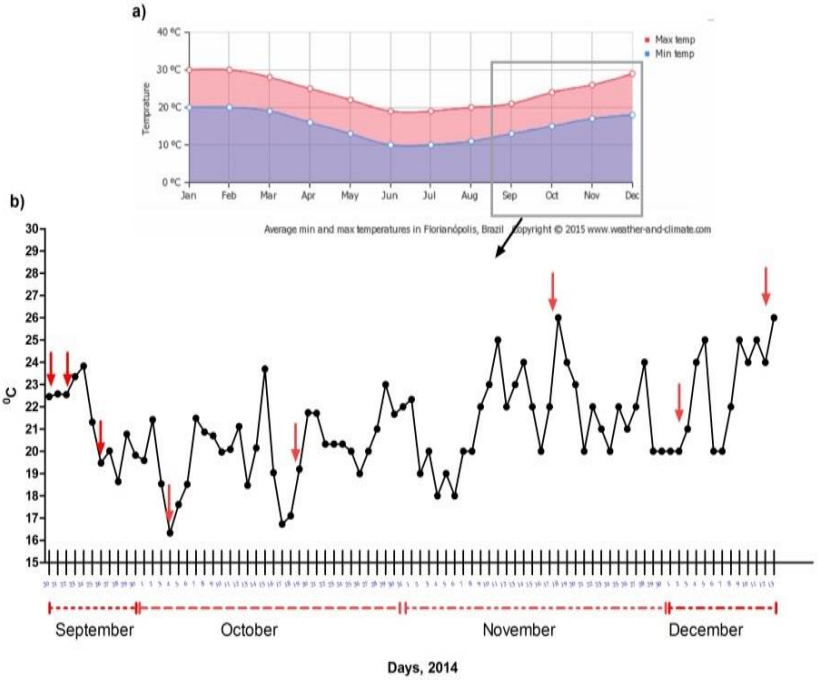


Figure 41: Temperature index along 85 days for a laboratory scale microcosm located in the open environment, a) Maximum and Minimum of temperature [year 2014, adapted from website: weather and climate.com], and b) temperature index for the experiment (85 days), where (↓) refers to the collection day for each microcosm.



3.4.2 Murine norovirus (MNV-1)

The time course of decay in natural environment for MNV-1 is shown in Fig. 42 (a). After 15 days, MNV-1 infectivity lost about 3 $logs_{10}$ (7.5×10^{1} PFU/mL and 5.9×10^{1} PFU/mL) either in water or sediment, respectively. After 30 days, we could not detect more infectious units by plaque assay in both matrices. These results pointed to the effect of temperature and natural U.V. light on viral stability and infectivity.

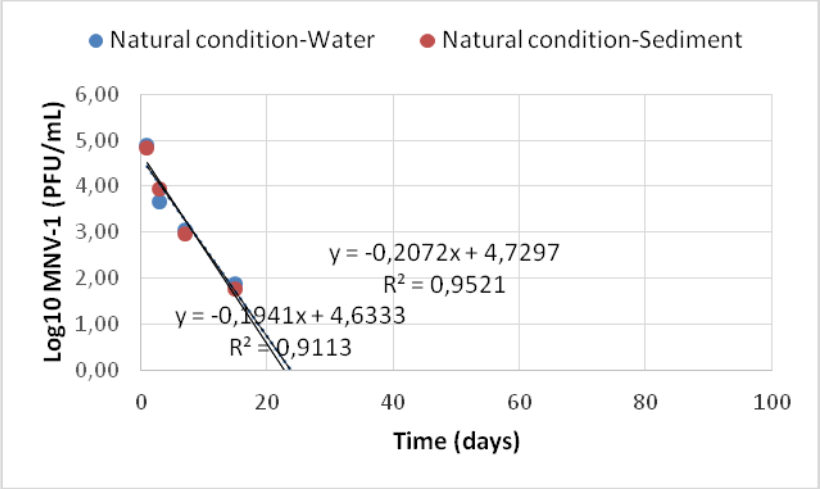
In dark system, in water column we could detect an average of 1.4×10^{3} PFU/mL of MNV-1 (about 1.7 log_{10} reduction) after 30 days and the infectivity was lost completely after 60 days. Contrary, MNV-1 was detected in the sediment along the time course of experiment until only 70 days with logs reduction of about 2.7 log_{10}. The log reduction values

were analyzed based on virus concentration detected after 24 h of settling process for virus after inoculation as shown in Fig. 42 (b).

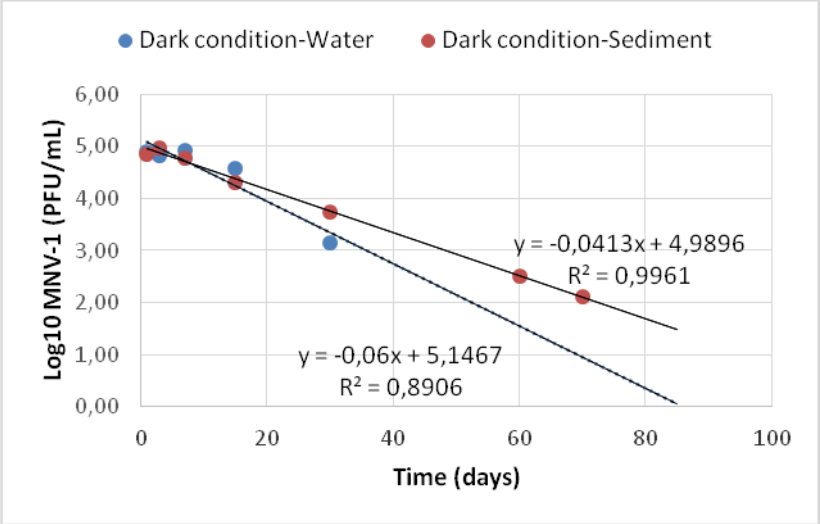
.

Figure 42: Survival and $\log_{10}$ reduction of MNV-1 in natural environment (a) and dark environment (b).

a)



b)



To estimate the required time to inactivate 90% of virus (T_{90}) in water and sediment either in natural or full dark environment, the obtained results from microcosms were adjusted. Linear regression was used for modeling (Microsoft Excel) as shown in Table 8.

All experiments exhibited a similar pattern of rHAdV viral settling over the initial 24 h after direct inoculation ($P<0.05$) as shown in Fig. 43. However, we could not note a clear settling for MNV-1 in both matrices as shown in Table (9).

Table 8: Rate of decay (T_{90}) for rHAdV and MNV-1 in water and sediment in laboratory scale microcosm

Virus	Water							
	Natural environment				Dark environment			
	$-k \pm$ SD	$T_{90} \pm$ SD	$P \leq 0.005$	$R^2 \pm$ SD	$-k \pm$ SD	$T_{90} \pm$ SD	$P \leq 0.005$	$R^2 \pm$ SD
rHAdV	0.129±0.003	7.7±0.156	$P \leq 0.05$	0.875±0.007	0.048±0.002	20.9±0.727	$P \leq 0.05$	0.801±0.016
MNV-1	0.151±0.009	6.7±0.436	$P > 0.05$	0.942±0.027	0.089±0.005	12±0.669	$P \leq 0.05$	0.945±0.007
	Sediment							
rHAdV	0.142±0.004	7±0.169	$P \leq 0.05$	0.829±0.043	0.045±0.002	22±0.915	$P \leq 0.05$	0.827±0.008
MNV-1	0.157±0.005	6.4±0.669	$P \leq 0.05$	0.946±0.033	0.05±0.001	18.6±0.534	$P \leq 0.05$	0.929±0.018

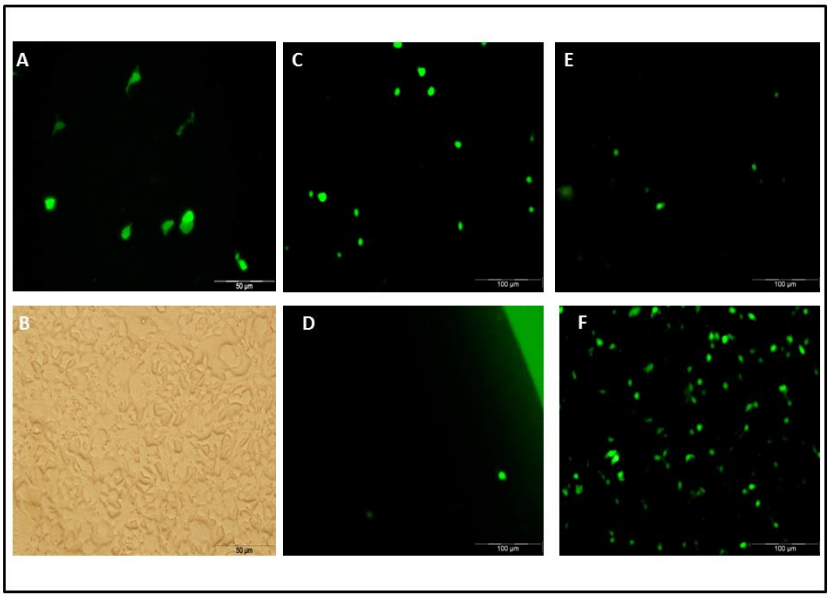
K: Constant decay rate; T_{90}: Time for decimal reduction of viruses; R^2: correlation coefficient of determination; SD: standard deviation.

Table 9: Infectious units for rHAdV and MNV-1 during initial 24 h of settling process

Sample type	rHAdV (FFU/ml)		MNV-1 (PFU/ml)	
	Zero	24 hr.	Zero	24 hr.
Water	$2.12 \times 10^{5*}$	$3.5 \times 10^{4*}$	5×10^{4}	7.8×10^{4}
Sediment	$1.33 \times 10^{4*}$	$1.73 \times 10^{5*}$	1.55×10^{5}	7×10^{4}

*Statestically significant

Figure 43: HEK 293A cells infected with rHAdV by fluorescence microscopy and light microscopy. Viral concentration of 5×10^{5} FFU/mL, rHAdV control (dilution 10^{-4}) (A), cell control (B), overlaying water (10^{-2}) at time zero (C), overlaying water (10^{-2}) after 24 h (E), sediment (10^{-2}) at time zero (D), sediment (10^{-2}) after 24 h (F), 100x magnification.



Source: Author

3.5 Sunlight effect on virus inactivation

From our results, it was confirmed that, under natural condition, there was a decay effect on viral stability and infectivity for both studied DNA and RNA viruses (rHAdV, and MNV-1), T_{90}-natural condition (predicted number of days to achieve a 1-$\log_{10}$ reduction) for rHAdV and MNV-1 were 7.7 and 6.7 days, respectively in water matrices. In sediment, T_{90}-natural condition was 7 and 6.4 days, respectively. Contrary, T_{90}-full dark condition for rHAdV and MNV-1 in water column was 20.9 and 12, respectively. While in sediment, T_{90}-full dark condition was 22.7 days for rHAdV as shown in Fig. 44 (a, b), and 18.6 days for MNV-1 as shown in Fig. 45 (a, b). Linear regression was used for modeling (Microsoft Excel, 2010) as shown in Table 8.

Figure 44: Linear regression model for natural and dark environment microcosm inoculated with rHAdV

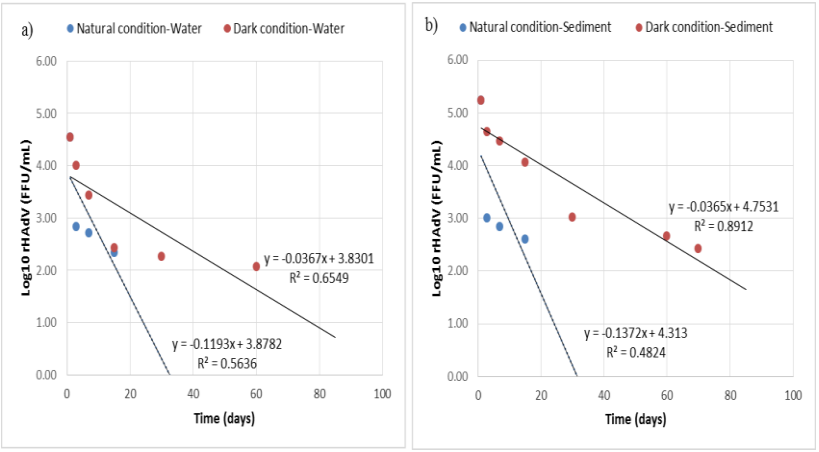
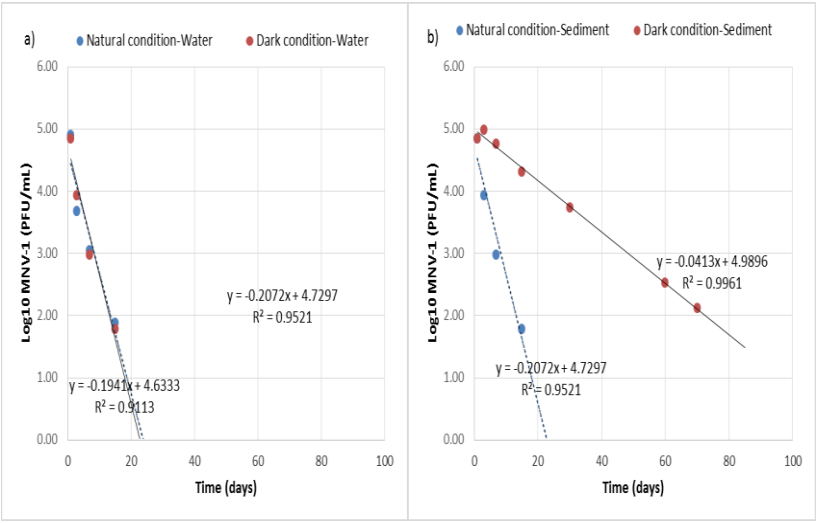


Figure 45: Linear regression model for natural and dark environment microcosm inoculated with MNV-1



4. DISCUSSION

This study examined the decay of two types of enteric viruses' rHAdV, and MNV-1 in dark and natural condition. Over 85 days of microcosms sampling period, we monitored the decay rate for two types of viruses rHAdV and MNV-1 (representing DNA and RNA genomes enteric viruses) that had been artificially inoculated with different initial concentrations at the same volume (1mL): 2×10^7 FFU/mL and 5×10^6 PFU/mL for rHAdV and MNV-1, respectively. For rHAdV, all experiments exhibited a similar pattern of settling over the initial 24 h after direct inoculation; while for MNV-1 the settling process was not uniform. This difference may reflect the intrinsic differences between adsorption processes for DNA and RNA viruses which rely on many factors such as size and shape of virus, surface charge and hydrophobic interactions, which may lead to variation in settling velocities and/or association with sediment particles (Wong et al., 2012).

Our results using microcosms, illustrated the extended persistence of viruses in sediments compared with overlaying water column in dark conditions. In the natural conditions, after the initial peak of settling, the concentration of rHAdV in the water and sediment decreased dramatically in a relatively short period of time 15 days which decreased by 2.2 and 2.6 orders of magnitude, respectively. After 30 days we could not detect more rHAdV infectious particles, since the FFU for rHAdV was below the limit of detection until the end of the experiment using fluorescence microscope.

The same happened for MNV-1 which decreased about 3 orders of magnitude after 15 days. After 30 days we also could not detect more MNV-1 infectious particles where PFU for MNV-1 was below the limit of detection until the end of the experiment using plaque assay.

However, in dark conditions, a similar time course of viral decay has been observed for rHAdV and MNV either in water or sediment samples, where infectious particles for rHAdV was detected until 60 and 75 days in water and sediment, respectively with $\log_{10}$ reduction decreased by 2.5 and 2.8 orders of magnitude in tested matrices, respectively. The FFU for rHAdV was no longer detectable after 70 and 85 days either in overlaying water or sediment, respectively. However, the reduction in infectivity of rHAdV reached 4.5 and 5.2 $\log_{10}$ in 70 and 85 days in both matrices (water and sediment), respectively.

While infectious particles for MNV-1 was detected only until 30 and 75 days in water and sediment, respectively. This decreased was 1.7 and 2.7 orders of magnitude in tested matrices, respectively. The MNV-1 PFU was no longer detectable after 60 days in water and after 85 days in

sediment where the reduction of infectivity of MNV-1 reached 4.8 log_{10} in each of them.

It is well known that viruses with a DNA double-stranded genome are more stable than those whose genome are RNA single-stranded like MNV-1 (ESPINOSA et al., 2008). However, the stability of MNV-1 which is ss-RNA, nonenveloped norovirus was comparable with stability of rAdV in current study (until 15 days of collection). A study done by KATPALLY et al., (2008), has reported that MNV may undergo a capsid maturation process, where the mature state may be more stable, which may account the stability of MNV-1 in the present study until 15 days in overlaying water and sediment samples with only 0.4 log_{10} reduction. Many studies in the literature are described regarding the stability of enteric viruses in different aquatic environment with conflicting results. Ground water: OGORZALY et al., (2010) they detected adenoviruses-2 throughout 120 days, the reduction of infectivity reached 2.4 log_{10} (T_{90}=35days) and 0.7 log_{10} (T_{90}=132days) at 20°C and 4°C (controlled temperature), respectively. CHARLES et al., (2009) they realized a rapid decrease in the infectivity of AdV-2 in a laboratory scale microcosm (4.2 log_{10} over the initial 21 days) during 2 years studies were undertaken on spiked groundwater at 12ºC nevertheless, they also observed that infectious HAdV-2 could still be detected over a total 364-day period (2 years). These differences with our results may be due to type of collection sample where OGORZALY and CHARLES depend on their studies on in *situ* groundwater microcosms, which considered to have a more stable composition and a higher microbial quality than those of surface waters that we used in the current study (Peri Lagoon-laboratory scale microcosm) and also due to the slow filtration of the water through different layers of soil and sediments, removing pathogenic microorganisms and many chemical compounds in case of ground water.

ENRIQUEZ et al., (1995), detected infectious HAdV serotypes 41 and 40 in artificially seeded tap, primary and secondary wastewater effluents that were kept in a Biological Oxygen Demand (BOD) incubator at either 4 or 15°C. In the case of tap water held at 4°C, after 55 days, the HAdV 41 and HAdV 40 lost nearly 0.5 and 1.0 log_{10}, respectively, with an estimated T_{99} value of 304 and 92 days for HAdV 41 and HAdV 40, In contrast, at 15°C, these agents lost almost 2 log_{10} at the same period. In primary wastewater, after 50 days at 4°C, HAdV 41 and HAdV 40 diminished about 2 and 2.5 log10 with an estimated T_{99} value of 44 and 48 days for HAdV 41 and HAdV 40. While at 15°C, 2.6

and 2.8 $\log_{10}$ for HAdV 41 and HAdV 40 with an estimated T_{99} value of 40 and 43 days for each respectively. In secondary sewage at 4°C for 50 days, the enteric adenoviruses 41 and 40 lost nearly 1.9 and 2.2 $\log_{10}$, respectively with an estimated T_{99} value of 47 and 58 days. However, at 15°C after 60 days, these agents (HAdV 41 and HAdV 40) lost almost 2.9 and 2.4 $\log_{10}$, respectively with an estimated T_{99} value of 45 and 43 days. Although, we believed that the viral survival studies in the literature are difficult to compare, as they are carried out under different conditions.

In general, the decay rates (*K*) for rHAdV observed in microcosms (containing overlaying water and sediment) that were exposed to sunlight in the natural condition (k =0.129 & 0.142; T_{90}= 7.7 & 7 days, resepectively) was higher than that observed in microcosms in the dark condition (k =0.046 and 0.018; T_{90}= 20.9 & 22.7 days) for water and sediment, respectively. The same was observed for MNV-1, where decay rates (*K*) in natural condition for water and sediment (k =0.151 & 0.157; T_{90}=6.7 & 6.4 days, respectively) was higher than that observed in case of dark condition (k =0.089 & 0.05; T_{90}=12 & 18.6 days). One possibility is that there is an interaction of temperature and U.V. sunlight leading to an increased rate of degradation in the open environment.

These results highlight the effect of sun light and direct temperature on the inactivation of these viruses in overlaying water and sediment. In this study we noticed that the decay rate for both viruses (rHAdV, and MNV-1) was rapid in the natural condition due to high temperatures noted along the experiment that occurred from September, 2014 to December, 2014. The results of this study corroborate with another previous study done by SIDHU; TOZE (2012), where they detect the decay rate for adenovirus in a laboratory microcosms established using 50-mL sterile polypropylene centrifuge tubes and incubated in the dark at 20°C in a refrigerated incubator using a groundwater samples, they noted a fastest rate of inactivation (k =0·047/day; T_{90}=21) of seeded adenovirus in the laboratory microcosms.

A study done by WATTS et al., (1995), reported the photocatalytic inactivation for survival of poliovirus 1 in secondary wastewater effluent using Batch reactors consisted of Pyrex glass dish. They concluded that the time required for two-log inactivation of poliovirus 1 was approximately 30 min under laboratory lights and sunlight. According to DAVIES-COLLEY et al., (1999) there are three independent mechanisms involved in sunlight-induced virus inactivation: direct photobiological damage, exogenous photooxidation

and endogenous photooxidation all of these mechanisms are catalyzed by photosensitizer (substances that initiate or catalyze photochemical reactions).

Natural organic matter (NOM) may act as a protective agent for viruses in the overlaying water, by which viruses can be adsorbed on it and also by attenuating the effect of natural sunlight by absorbing shorter wavelength (KELBLE et al., 2005). However SUN et al., (2016) showed that the presence of Natural organic matter in the aquatic matrices (NOM) may play a role on enhancing viral inactivation depending on its composition and concentration in the matrices. NOM is natural photosensitizer in surface waters which can contribute in the production of reactive oxygen species (ROS), However HAdV has the ability of DNA repair after damaged but this later (ROS) could damage the HAdV capsid protein without the possibility to repair (CANONICA et al., 1995; SILVERMAN et al., 2013).

However, it should be taken in consideration that, unlike in the current study using microcosms as a model considers only dark and natural light effect, the natural environment is not static and many factors such as turbidity because of suspended solids associated with storm water events and winds, concentration of microorganisms and tidal movement, all of these factor may significantly reduce the penetration of U.V. sunlight exposure in overlaying water and will influence in microorganism decay rate. Our finding agreed with another study done by PEDLEY et al., 2006 which demonstrated that the stability of infectivity in viruses is known to be greater in specific conditions: low temperatures, no light and limited predatory populations. Regarding to the microbiota, WETZ et al., (2004) demonstrated the poliovirus survival rate in unfiltered and filtered seawater, where they noted that poliovirus survive more in filtered water than in unfiltered water.

Though, nature of samples is an important issue that could influence the activity of the indigenous micro-organisms, and their virucidal activity as well as production of ROS, which ultimately influence decay rates of enteric virus independently of natural sunlight effect.

5. CONCLUSION

-In general, these results showed that the prolonged survival of viruses in overlaying water and sediment in specific conditions, proved by infectivity assays, can indicate a health hazard,

-The presence and persistence of these viruses in sediment layer (particularly in sediments consisting of small particle size and high organic carbon as detected in this study) may indicate an increased risk of infection because of the persistence and possible resuspension of these pathogens during natural turbulence or human activity,

-The results indicated that natural UV sunlight (UVA/visible light) could directly cause virus inactivation.

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GENERAL CONCLUSIONS

1- Analysis of water and sediment samples from the Peri Lagoon and Sangradouro River revealed that this region is under anthropogenic impact of human sewage discharge, and there is a possible contamination even by the human sewage or by the normal human activities in this area. This contamination poses a threat to the population because the Peri Lagoon is the only water source used by CASAN to supply drinking water for local population,

2- It was clear from this work that sediment plays an important role as a reservoir for viruses with high concentrations,

3- The lack of studies related to detection and survival of enteric viruses in sediments make this work relevant to push the decision makers to consider combined analyses of water and sediments for reliable public health risk analysis of recreational and lagoon waters,

4- None of the physical-chemical parameters showed sufficiently consistent significant correlations with the presence or infectivity of viruses,

5- Due the wide distribution of HAdV in water and sediment samples during our study along two year of surveillance, we can easily say that HAdV should be viewed as an index for the presence of human enteric viruses and other human faecal pollution for better protection to the public health,

6- Somatic coliphage showed no positive correlation with the presence of other enteric viruses either in water or sediment samples, so we can say that it is not a good indicator or surrogate for the presence of enteric viruses in the aquatic ecosystem,

7- Infectivity assay for these viruses (HAdV in present study) must be addressed for a good and comparable estimation of the risk associated to the consumption of contaminated water and food irrigated with contaminated water,

8- It was clear, from the laboratory scale microcosm, that the persistence of these infectious viruses are prolonged in the aquatic environment,

9- Also our study supports the idea about the photodegradation of viruses either adsorbed to sediment particle or suspended in the overlaying water column. However, the dark condition in deep water can play an important role in the prolonged infectivity of these viruses in the aquatic environment,

10- At all, transmission of these human enteric viruses is linked directly to insufficient treatment of sewage waste by wastewater treatment facilities so it must be taken in consideration.

PROSPECTS

The results obtained from the present study provided an important focus on the fate of these pathogens in the aquatic environment especially in sediment as a main refuge for these pathogens based on the processes of virus-solid adsorption and settling. Also, the importance of U.V. sunlight as an alternative method for disinfection of these pathogens in water in addition to chlorine, ozone and other traditional methods.

APPENDIX: Articles published corresponding to other collaborative activities undertaken during the doctoral period (2012-2016):

Article 1 (cooperation article).

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Utility of specific biomarkers to assess safety of swine manure for biofertilizing purposes

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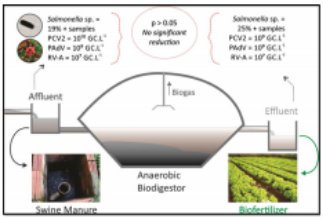
HIGHLIGHTS

- Enteric viruses and Salmonella spp. persist even after the anaerobic biodigestion of liquid swine manure
- PCV2, PAdV and RVA genomes were positive in 77.5%, 60% and 37.5%, of the samples respectively
- Salmonella spp. was found in 40% of the samples collected during the summer and in 15% during the winter
- It is necessary to establish more efficient sanitization methods for biofertilizer purposes from swine manure

GRAPHICAL ABSTRACT



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ABSTRACT

Swine production is an important economic activity in Brazil, and there is interest in the development of clean production mechanisms to support sustainable agro-industrial activities. The biomass derived from swine manure has good potential to be used as a biofertilizer due to its high nutrient concentration. However, the land application of manure should be based on safety parameters such as the presence of pathogens that can potentially infect animals and people. This study was designed to assess the presence of porcine circovirus-2 (PCV2), porcine adenovirus (PAdV), rotavirus-A (RV-A) and *Salmonella* spp. in liquid manure, as well the infectivity of two genotypes of circovirus-2 (PCV2a and PCV2b) present in liquid manure. Three swine farms were evaluated: 1) a nursery production farm (manure analyzed before and after anaerobic biodigestion), 2) a grow-finish production farm (analyzed before and after anaerobic biodigestion), and 3) a second grow-finish production farm (raw manure-affluent). PCV2, PAdV and RV-A were present before and after anaerobic biodigestion (either affluent or effluent) at all farms. *Salmonella* spp. were detected at farm 1 (affluent and effluent) and farm 3 (raw manure-affluent) but not farm 2 (affluent and effluent). When the ability of the anaerobic biodigestion process to reduce viral concentration was evaluated, no significant reduction was observed ($P > 0.05$). Both the PCV2a and PCV2b genotypes were detected, suggesting viral co-infection in swine production. The results revealed infectious PCV2 even after anaerobic biodigestion treatment. The presence of *Salmonella* spp. and enteric viruses, especially infectious PCV2, in the final effluent from the anaerobic biodigester system suggests that the process is inefficient for pathogen inactivation. Due to the prevalence and infectivity of PCV2 and considering the successful use of molecular methods coupled to cell culture for detecting

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Review article (1)

Virus Reviews and Research
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journal homepage: www.vrrjournal.org.br/

Review Article

ENTERIC VIRUSES AS CONTAMINANTS AND BIOINDICATORS IN ENVIRONMENTAL SAMPLES

Fongaro, G.[1]; Silva, H.D.[2]; Elmahdy, E.M.[1]; Magri, M.E.[1]; Schissi, C.D.[1]; Moreira, M.[2]; Lanna, M.C.S.[3]; Silveira-Lacerda, E.P.[2]; Barardi, C.R.M.[1]*

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ABSTRACT

Gastroenteritis, diarrhea, and other diseases can be caused by enteric viruses transmitted by fecal-oral route. Human adenoviruses (HAdV), rotavirus A and C (RVA and RVC, respectively), hepatitis A and E virus (HAV and HEV, respectively), human astroviruses (HAstV), human noroviruses (HuNV) and enteroviruses (EV) are, among the enteric viruses, the most frequently detected in environment samples. These viruses are usually introduced into aquatic environments by human, industrial, or agricultural activities and are widely distributed all over the world. They have the common characteristics to be structurally stable and can also absorb to solid particles and biofilms, thereby protecting themselves from inactivating factors. This revision aimed to present and discuss: i) most relevant enteric viruses for human and animal health; ii) enteric viruses as contaminants and bioindicators in environmental samples; iii) molecular and cell culture methods for enteric virus detection; iv) use of enteric viruses for microbial risk assessment. Impacts of enteric viruses on environment and the potential use as bioindicators of the sanitary security, such as presence and infectivity studies were discussed as development of new tools for disinfection, monitoring, risk modeling and management, among other studies.

Keywords: enteric viruses; bioindicators; contaminants; environmental samples
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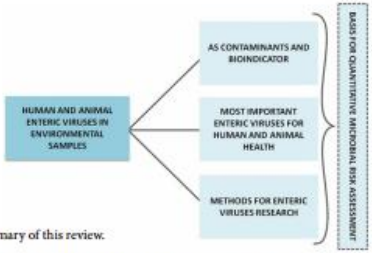

Figure 1. Schematic summary of this review.

1. ENTERIC VIRUSES AS CONTAMINANTS AND BIOINDICATORS IN DRINKING AND WASTEWATER

According to the World Health Organization (WHO), it is estimated that 663 million people worldwide still use improper drinking water sources, including unprotected wells and springs and surface water (WHO, 2015).

World legislations for enteric virus vigilance in environment matrices are scarce. The Center for Disease Control and Prevention-(CDC-USA) recommends Hepatitis A virus surveillance in foods (CDC, 2005); The Centre for Environment, Fisheries & Aquaculture Science

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Review article (2)

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Monitoring viruses in environmental samples

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ABSTRACT

Environmental virology is a field that has grown in the last 50 years. Because viruses can be transmitted by contaminated water, this work is of special concern for human and animal health. Enteric viruses, mainly consisting of adenovirus, polyomavirus, norovirus, hepatitis A and E, rotavirus A and C, and porcine circovirus, can be found in environments contaminated with human and animal feces. Despite the importance of this issue to health and the recommendations of various studies to use viruses as environmental contamination markers, viruses are not routinely monitored in drinking water. This review aims to contribute to the case for the inclusion of viral surveillance in tests of water and food quality. We have gathered information about important food- and waterborne viruses implicated in outbreaks, the main methods available to concentrate and detect these viruses from different environmental matrices, and also the current status of legislation in Europe, the United States and Brazil

Key words: *Environmental samples, enteric viruses, concentration methods, detection methods, legislation*

1.0 INTRODUCTION

Viruses are of special concern for human and animal health due to their wide distribution, rapid transmission, and economic impact. Enteric viruses are transmitted through the fecal-oral route by the consumption of contaminated water, food or aerosols. This transmission is mainly related to untreated or badly treated sewage discharged into the environment.

The field of environmental virology emphasizes the importance of viruses transmitted by diverse water sources and foods.

These studies began in the middle of the twentieth century, when human outbreaks caused by contaminated water and food were first reported (Melnick, 1947). Since then, viruses transmitted by aquatic environmental routes have been investigated all over the world, in diverse environments. In particular, viruses of human and animal interest (such as adenoviruses, polyomaviruses, noroviruses, hepatitis A and E, rotaviruses A and C, porcine circoviruses, bacteriophages, and others) have been investigated in water matrices, sewage and raw foods such as mollusks and vegetables (Bofill-Mas *et al.*, 2000; Enriquez *et al.*, 1995; Rigotto *et al.*, 2010; Viancelli *et al.*, 2012).

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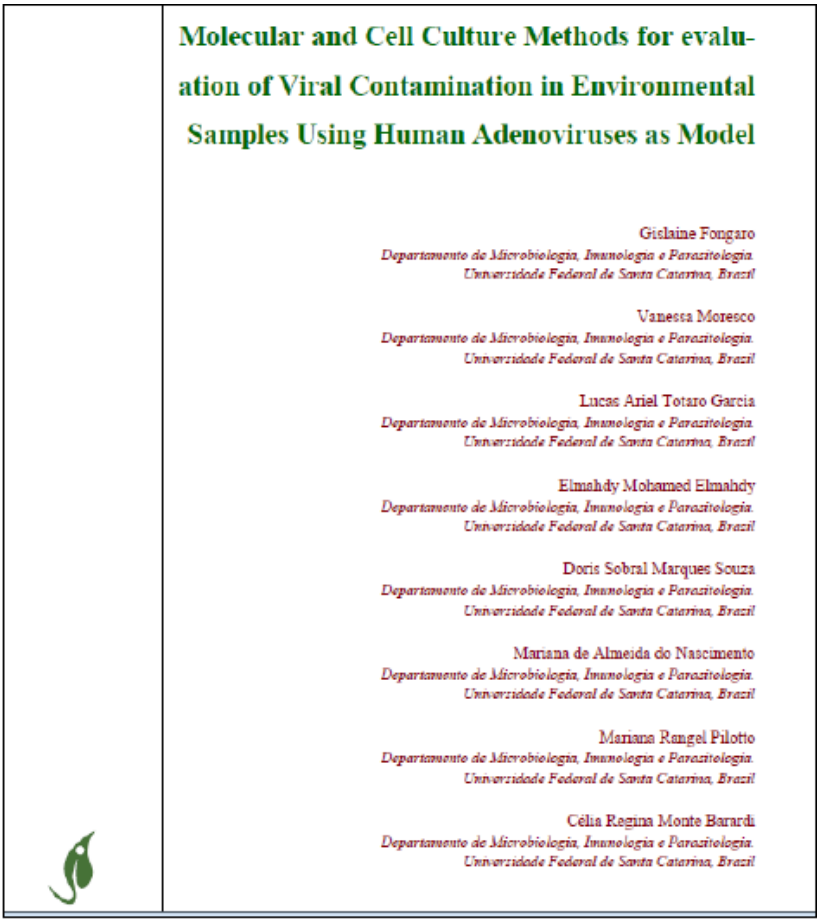

Molecular and Cell Culture Methods for evaluation of Viral Contamination in Environmental Samples Using Human Adenoviruses as Model

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International Conference in France to present the results of the PhD thesis (chapter II)

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Le Pecq, March 17th, 2015

Invitation to the "7th International Water & Health Seminar for PhD Students", Cannes, France, June 29th – July 1rst, 2015

Dear Mr Elmahdy Mohamed Elmahdy Ibrahim,

Following the acceptance of your application by the scientific committee, I have the pleasure to confirm that you are invited to present your PhD work at the next edition of the "International Water & Health Seminar for PhD Students", that will take place in Cannes, France, from June 29th to July 1rst, 2015.

You will be hosted at ADOSOM Windsor Residence, 16 avenue Windsor, 06406 Cannes. Accommodation and food expenses will be covered by our company, from June 28th to July 2nd. However, travel expenses from Brazil to Cannes will be at your own charge.

Looking forward to meeting you in Cannes

Jean-François Loret
Director, Health & Environment
Research Program

Profa. Célia Regina Monte Barardi
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Le Pecq, July 31, 2015

CERTIFICATE OF ATTENDANCE

I the undersigned, Jean-François Loret, certify that Elmahdy Mohamed Elmahdy Ibrahim attended the seventh International Water & Health Seminar in Cannes, France, from June 29th to July 1st, 2015, where he made an oral presentation entitled "Quantification of waterborne human enteric viruses and bacteriophages in surface water and sediments in Florianapolis river and lagoon".

Jean-François Loret
Director, Health & Environment Research Program