Universidade de Lisboa

Faculdade de Farmácia

Adenoviruses' inactivation mechanisms by gamma radiation

Catarina Rua Palma

Dissertation supervised by Dra. Sandra Cabo Verde and co-supervised by Professor Doctor João Gonçalves.

Biopharmaceutical Sciences Master

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Abstract

Adenovirus is the most prevalent enteric virus in waters and inefficiently removed by conventional wastewater treatments leading to public health concerns. The main goal of this thesis project was to study the adenoviruses' inactivation mechanisms by gamma radiation treatment, through the evaluation of the effects on genome and capsid proteins. The insights on the inactivation of enteric virus via gamma radiation aim to contribute to develop alternative water disinfection treatments. This project addressed ONU sustainable development goals, namely ensure availability and sustainable management of water and sanitation for all.

The HAdV-5 was inoculated in PBS and wastewater substrates and irradiated in a Cobalt-60 irradiator at five gamma radiation doses (1.5, 3, 4, 15 and 25 kGy). The amplification of long regions of HAdV-5 genome, the viral proteins abundance and antigenicity and, the infectivity were evaluated for all tested doses. Despite the similar results for the amplification of fragments of viral genome from virus suspensions on PBS and wastewater irradiated at 4 kGy; the proteins abundance and antigenicity, as well as, the viral infectivity were higher for the wastewater substrate. The viral proteins were degraded with different sensitivity to gamma radiation. The HAdV-5 structural proteins of capsid, hexon, penton and fiber showed higher radioresistance to gamma radiation comparatively to other viral proteins. The relative binding of a monoclonal antibody to the hexon antigen on PBS samples was only 17%, contrasting with 88% for wastewater samples. The virus titer $\log_{10}$ reduction when suspended in PBS and wastewater was 6.5 and 5.0, respectively. These differences can be explained by a protective effect of the organic matter present in the wastewater substrate against the indirect effects of gamma radiation. According to the obtained results, the HAdV-5 DNA degradation could be considered the main factor for viral infectivity decreases and virus inactivation. The outputs of this work evidenced that for both substrates a treatment with 4 kGy gamma radiation dose could produce a viral inactivation superior to the conventional wastewater treatments, removing >99,99% of HAdV-5. The understanding of viral inactivation mechanisms by ionizing radiation will provide new insights for application of this technology on wastewater treatments.

Keywords: enteric virus; adenovirus; gamma radiation; wastewater treatment

Resumo

Um dos objetivos da ONU para o desenvolvimento sustentável é assegurar a disponibilidade e gestão sustentável da água e saneamento para todos. Os vírus entéricos representam um desafio societal mundial ao nível da contaminação de águas para consumo humano. Os vírus entéricos são excretados pelas fezes, transmitidos pela via oral-fecal e replicados no trato gastrointestinal humano. As doenças transmitidas através de água contaminada representam uma mortalidade de 1.5 a 12 milhões de pessoas por ano, das quais uma grande parte é causada por infeções provenientes de água potável contaminada.

A constante evolução e variação de serotipos virais conferem aos vírus entéricos uma elevada plasticidade. Em águas contaminadas podem ser encontrados até 150 tipos de vírus entéricos. A elevada diversidade, resistência e infecciosidade conferem a estes vírus uma elevada persistência no meio ambiente. Os vírus entéricos causam infeções no sistema nervoso central, gastroenterites, conjuntivites e doenças respiratórias.

Os adenovírus foram considerados um indicador do nível poluição das águas devido à sua elevada resistência a fatores químicos e físicos. As infeções causadas por adenovírus têm uma elevada incidência em crianças e indivíduos imunocomprometidos. Os vários serotipos deste vírus são associados a diferentes infeções, tais como pneumonia (serotipos 3,5 e 7), conjuntivite (serotipos 8,19 e 37) e gastroenterite (serotipos 40 e 41). Nas águas recreativas, o risco de infeção é estimado em cerca de 1 em 1000 para uma única exposição. O principal fator da complexidade e resistência dos adenovírus é considerado a presença de uma cápside cujas principais proteínas são o hexão (pII), pentão (pIII) e a fibra (pIV) que se interligam numa rede complexa. Um dos objetivos deste estudo foi contribuir para a implementação de novas tecnologias de radiação ionizante para o tratamento de efluentes, tendo em conta a documentada ineficiência dos métodos tradicionais no tratamento de vírus entéricos.

As tecnologias de radiação são um processo de desinfecção seguro, não térmico, que não recorre a substâncias químicas que podem deixar resíduos, tendo a vantagem do tratamento poder ser realizado diretamente no produto final. A radiação gama emitida pelo radioisótopo cobalto-60 (Co-60) apresenta um elevado poder de penetrabilidade em materiais líquidos e sólidos. Esta tecnologia é globalmente utilizada na esterilização de produtos médicos, produtos alimentares e biológicos conservando as suas caraterísticas e valor nutricional. A inativação microbiana por radiação gama ocorre pelo efeito direto dos fotões no genoma dos microrganismos e indiretamente pela formação de radicais livres provenientes da radiólise da água. Estes radicais por sua vez, podem interagir com o material genómico e produzir quebras simples ou duplas na cadeia de ADN. A degradação do ADN pelo efeito direto e indireto da radiação gama é descrito como o principal fator para a inativação de microrganismos. Os efeitos da radiação podem também ter impacto ao nível das proteínas, nomeadamente na quebra das interações hidrofóbicas e ligações covalentes e não covalentes, responsáveis pela estrutura da proteína.

A constituição do meio e a atmosfera de irradiação tem uma elevada influência na resposta dos microrganismos à radiação gama. De uma forma geral, a presença de proteínas, carbohidratos e álcoois num dado substrato protege contra os efeitos da radiação, na medida em que estas moléculas estabelecem ligações com radicais livres impedindo assim a degradação do genoma microbiano. A presença de nitritos, nitratos e quinonas tem o efeito contrário, aumentando a eficiência do tratamento por irradiação. O conteúdo em água e a atmosfera rica em oxigénio são também fatores que sensibilizam os microrganismos à radiação gama. Adicionalmente, o tamanho e complexidade dos microrganismos podem também influenciar a resposta à radiação ionizante, em termos gerais os microrganismos de maior tamanho como as bactérias e fungos, são mais sensíveis à radiação comparativamente com os vírus que são considerados mais radioresistentes.

O estudo dos principais mecanismos de inativação de vírus, nomeadamente de adenovírus (adenovírus humano serotipo 5, HAdV-5), pela radiação gama foi o objetivo principal deste estudo. Deste modo, os efeitos ao nível do genoma viral, das proteínas e da infecciosidade viral foram avaliados em dois substratos (PBS e efluente municipal antes do tratamento terciário) de forma a mimetizar possíveis cenários onde o tratamento por radiação ionizante poderá ser aplicado como método de desinfeção de vírus entéricos humanos. As suspensões de HAdV-5 em PBS e efluente foram tratadas por radiação gama às doses 1.5, 3, 4, 15 e 25 kGy e subsequentemente analisadas comparativamente com suspensões não irradiadas.

Os efeitos da irradiação ao nível do ADN viral foram estimados por amplificação de dois fragmentos longos do genoma de HAdV-5 por PCR, 1000 pb e 10000 pb. O fragmento de 1000 pb do genoma de HAdV-5 foi amplificado em todas as amostras (não irradiadas e irradiadas), enquanto que a amplificação do fragmento do genoma de maior dimensão 10000 pb não foi detetada para as suspensões virais irradiadas a partir dos 4 kGy. Estes resultados sugerem que a potencial fragmentação do ADN viral por radiação gama deverá ser maioritariamente em fragmentos superiores a 1000 pb, de forma a permitir a amplificação de fragmentos do genoma desta dimensão. Com base nos resultados obtidos e na metodologia aplicada, não foi detetada a influência do substrato de irradiação (PBS vs. efluente) na amplificação de fragmentos do genoma de HAdV-5.

Os efeitos da radiação gama nas proteínas virais foram analisados por SDS-PAGE, western-blotting com anticorpo policlonal anti-adenovirus e ELISA com anticorpo monoclonal anti-hexão. A abundância e antigenicidade das proteínas virais decresceu com o aumento da dose de radiação gama aplicada às suspensões virais, contudo as proteínas estruturais pII (hexão), pIII (pentão) e pIV (fibra) demonstraram por western-blotting uma elevada radioresistência, sendo detetadas nas suspensões virais irradiadas a 25 kGy.

O efeito do substrato na irradiação foi evidenciado ao nível das proteínas virais. As suspensões virais em PBS apresentaram uma maior degradação proteica comparativamente com as amostras em efluente. A carência química de oxigénio (CQO) é superior no efluente, existindo assim uma maior quantidade de matéria orgânica suscetível à oxidação que confere uma proteção adicional contra os efeitos indiretos da radiação gama (radiólise).

A antigenicidade da proteína mais abundante da cápside de adenovírus, o hexão, foi igualmente avaliada para os dois substratos, PBS e efluente, após o tratamento por radiação gama. Para a dose de tratamento de 4 kGy, a percentagem relativa de ligação ao antigénio foi de 17% para as suspensões virais em PBS e de 88% para as amostras em efluente. A infecciosidade das amostras foi medida através da redução do log_{10} do título viral para ambos os substratos. Considerando a referida dose de radiação de 4 kGy verificou-se uma redução de 6.5 e 5.0 log_{10} da carga viral nas suspensões em PBS e efluente, respetivamente. Os resultados obtidos indicaram assim uma influência do substrato de irradiação na inativação de adenovírus humano. Relativamente aos mecanismos de inativação por radiação gama, a análise global sugere que a degradação do genoma poderá ser o principal fator, tendo em conta que não foi detetada amplificação de longos fragmentos do genoma associado ao decréscimo da infecciosidade viral nas amostras irradiadas às doses mais elevadas, mas foi observada a presença das principais proteínas estruturais.

Os estudos efetuados documentaram a atividade virucida da radiação gama, indicando ser uma tecnologia de desinfeção promissora nomeadamente no tratamento de efluentes. Os tratamentos de efluentes convencionais apenas reduzem a concentração viral em 2 a 3 log_{10} PFU/ml sendo necessária, segundo a Agência de proteção ambiental, uma redução de pelo menos 4 log_{10} PFU/ml (99,99% de remoção viral). Considerando, os resultados obtidos nas amostras em efluente irradiadas a 4 kGy verificou-se uma eficiência de inativação superior.

Palavras-Chave: vírus entéricos; adenovírus; radiação gama; tratamento de efluentes.

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List of Abbreviations

AdV: Adenovirus

ADP: Adenovirus death protein

CAR: Coxsackie-adenovirus receptor

COD: Chemical oxygen demand

DBP: DNA binding protein

EV: Enterovirus

HAdV: Human adenovirus

HAV: Hepatitis A

HEV: Hepatitis E

ITR: Inverted terminal repetition

MLP: Major late promoter

MLTU: Major late transcription unit

MTOC: Microtube organization center

NoV: Norovirus

NPC: Nuclear pore complex

Ori: Origin of replication

Pol: DNA polymerase

pTP: Pre-terminal protein

RV: Rotavirus

TP: Terminal protein

VA-RNA: Virus-associated RNA

Introduction

Introduction

Viruses are the smallest microorganisms known, simply represented by a genome consisting of either RNA or DNA, surrounded by a protein capsid and in some cases by a lipidic membrane (HR. 1996). Viruses are obligate parasites depending on specific hosts for reproduction. They can be classified in lysogenic or lytic virus, depending on replication cycle type. In lysogenic cycle, the viral genome is incorporated by genetic recombination within chromosome host, and lytic cycle results in a quickly viruses production and cells destruction without chromosome incorporation. Viruses can infect all life forms, however, most of the viruses infect only specific cells within a host with different types of susceptibility (Flint 1994).

Human viruses that can multiply in the human gastrointestinal tract are denominated enteric viruses (Bisseux et al. 2018). Enteric viruses are excreted in feces and transmitted by the fecal-oral route (Fongaro et al. 2015). It has been suggested that more than 150 types of enteric virus may be present in contaminated waters (Wong et al. 2012). Infection by enteric virus can cause gastroenteritis, heart anomalies, meningitis, conjunctivitis, hepatitis and respiratory diseases (Swenson, P. D., Wadell, A., Allard, A., Hierholzer 2003). In sensitive populations such as children, the elderly, and the immune-compromised, waterborne viral infections can be fatal (Ziqiang Yin 2015).

The introduction of the enteric virus in aquatic environments results from human activities such as incorrect use of septic systems, urban and agricultural runoff, and in case of marine waters, vessel wastewater discharge for sea (Fong and Lipp 2005). Subsequently, the pathogenic viruses are transported by rivers, groundwater and recreational waters with possible accumulation in shellfish and, in irrigated vegetables and fruits (M V Yates, Gerba, and Kelley 1985; Rose et al. 1987). Statistics of waterborne disease reflect a growing global burden of infectious diseases from contaminated drinking water and a mortality of 1.5 to 12 million people per year (Ziqiang Yin 2015; Gleick 2002).

Human enteric viruses include various genera such as adenovirus (AdV), enterovirus (EV), norovirus (NoV), rotavirus (RV), hepatitis A (HAV) and E (HEV) viruses. The AdV and EV have been considered as human pollution indicators for water pollution sources identification (Hundesa et al. 2006). Most of these viruses have low infectious doses, high resistance and persistence in the environment, and are released in very high numbers in feces (Haas et al. 1993; Fong and Lipp 2005) . In comparison with pathogenic bacteria at similar exposures, the risk of infections by viruses in drinking water is 10 to 10.000-fold greater (Haas et al. 1993). Considering these facts, inadequate disinfection of fecally contaminated drinking water could lead to viral outbreaks.

Adenovirus

Adenoviruses infection is a worldwide problem and occurs throughout the year. More than 50% of the population is naturally infected by adenovirus and the infection is usually acquired during childhood due to lack of humoral immunity (Lynch and Kajon 2016; Foy, H.M. and Grayston 1976). Furthermore, in immunosuppressed persons, the respiratory failure develop are represented for 10-30% of cases (Kojaoghlanian, Flomenberg, and Horwitz 2003). Infections result from exposure to infected individuals (e.g. inhalation of aerosolized droplets, conjunctival inoculation, fecal-oral spread), acquisition from exogenous sources (e.g. pillows, linens, lockers), or reactivation (Doerfler W. 1996). After primary infection, the incubation period ranges from 2 to 14 days (Ison 2006). The adenoviruses latent form may reside in lymphoid tissue, renal parenchyma or other tissues for prolonged periods (Kojaoghlanian, Flomenberg, and Horwitz 2003).

One of the earliest reports of the adenovirus isolation was from a cell line of adenoid tissue in patients with respiratory infections. The authors found a correlation between human adenoid primary cells degeneration and viral replication (Hllleman and Werner 1954). Human adenoviruses are classified in the *Mastadenovirus* genus which contains 52 serotypes divided into 7 species (A-G) based on immunological distinctiveness and sequence (Seto et al. 2011). They have a wide range of hosts as non-human primates, mouse, dog, pig, chicken, and humans (Harrach et al. 2011). Human adenovirus cause diseases as ocular infections, gastroenteritis, respiratory disease, encephalitis, genitourinary infection, and pharyngoconjunctival fever (Swenson, P. D., Wadell, A., Allard, A., Hierholzer 2003; Ziqiang Yin 2015). The serotypes are associated with different conditions: pneumonia (HAdV serotypes 3,5 and7), eye infections (serotypes 8,19 and 37) and gastroenteritis (serotypes 40 and 41) (Norrby et al. 1976).

Adenoviruses may survive extended periods of time outside host cells, resisting to physical and chemical agents as well as adverse pH conditions. Infectivity is optimal between pH 6.5 and 7.4, although the viruses can withstand pH ranges between 5.0 and 9.0. Additionally, adenoviruses are heat-resistant and remain infectious after freezing (A. 2009).

1.1. Virion structure

Adenoviruses are lytic non-enveloped viruses and manifest icosahedral symmetry (20 triangular surfaces and 12 vertices) with approximately 70-100 nm in diameter containing a linear double-stranded DNA (Fig.1) (HORNE, R.W. et al. 1959; Nemerow 2000; Flint 1994; M. Horwitz 1996).

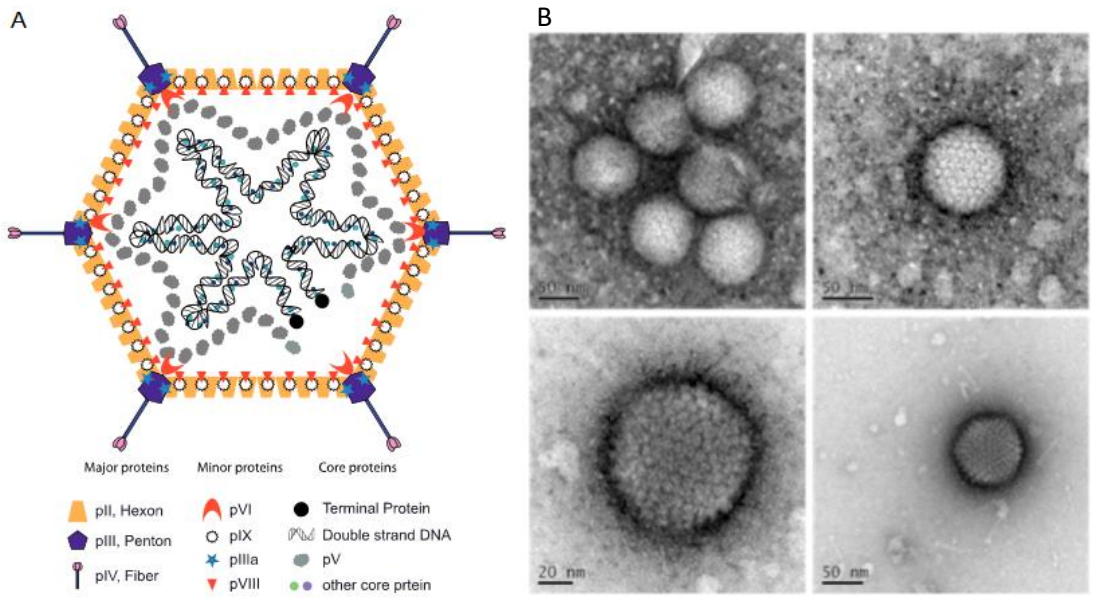

Figure 1- Human adenovirus structure. *(A) Schematic representation of HAdV and (B) Electron microscopic images* (Yu 2013; Russell 2007).

The viruses are stable in diverse temperatures for long periods of time, due to the absence of a lipoprotein membrane. Also, they are unsusceptible to organic solvents, such as ethanol and ether (Wassermann 1962; M. S. Horwitz 1990). The virus particle contains 11 structural proteins (pII, pIII, pIIIa, pIV, pV, pVI, pVII, pVIII, pIX, pX, and TP) numbered according to the size through SDS-PAGE migration (Fig.2) (Russell 2007).

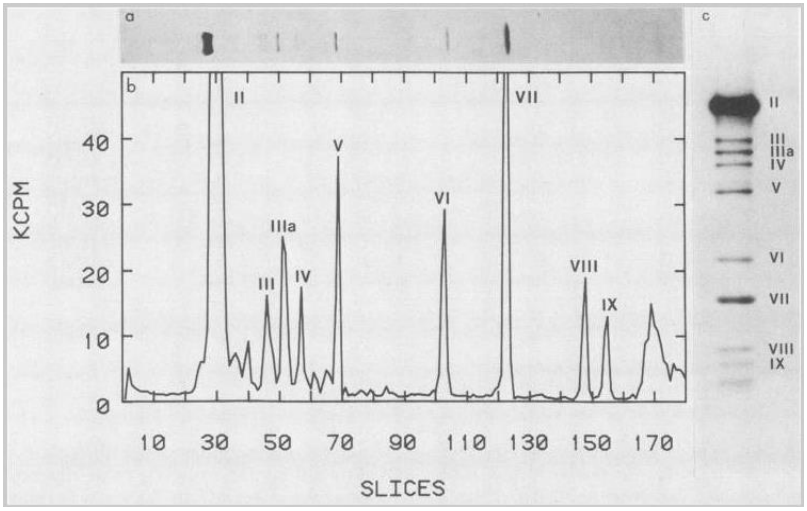

Figure 2- HAdV polypeptides separation. *Stoichiometric analysis performed with [35S] methionine as a radiolabel in a 220-mm linear 10 to 17.5% SDS-polyacrylamide gradient gel* (van Oostrum and Burnett 1985).

The protein coat or capsid comprises 7 of the 11 total proteins (pII, pIII, pIV, pIIIa, pVI, VIII, and pIX) and contains 252 sub-units, the capsomers, of which 240 are trimeric hexons (pII) and 12 are pentons (pIII and pIV) (Norrby et al. 1976; WADELL et al. 1980; van Oostrum and Burnett 1985). The hexon is the largest and most abundant structural protein in the adenovirus capsid (Celia A Toogood et al. 1989). The pentons form the vertices of the icosahedron and sustain a glycoprotein fiber (pIV) that projects outward. There are two well-understood functions associated with the fiber protein, the structural role in the viral capsid and the interactions with cellular adenovirus receptors (Wu et al. 2003). The fiber has three structural domains: the proximal tail (N-terminal), the shaft in the middle part, and the distal head (C-terminal) or knob domain responsible for recognition of cell surface receptors for the initial attachment to the host cell (Gaden et al. 2004). In addition, the knob domain is involved in intracellular trafficking, endosomal release and virus maturation (Vellinga, Van der Heijdt, and Hoeben 2005). The capsid also contains minor coat proteins (pIIIa, pVI, pVIII, and pIX) involved in capsid stabilization and flexibility of the viral particle (Martín 2012), and the core proteins (pV, pVII, pX and pTP) associated with the viral double-stranded DNA molecule, forming a condensed core structure (Wiethoff et al. 2005). Also, virions contain cysteine proteinase responsible for the cleavage of structural proteins during viral maturation.

1.2. Replicative cycle

Adenovirus infects both dividing and non-dividing cells in a wide range of cell types. The infection initiates with the attachment between the adenoviral fiber knob domain and the cell surface receptor (Yu 2013). The primary receptors vary according to HAdV types. HAdV from species C serotypes, such as type-2 and type-5, as well as from species A, D, E and F recognize the Coxsackie-adenovirus receptor (CAR) (Bergelson et al. 1997). After cell receptor binding by the virus fiber knob domain, the penton base binds to integrin molecules on the cell surface. This binding stimulates actin polymerization followed by endocytosis of the virus particles via clathrin-coated pits (Wu and Nemerow 2004). The decreasing pH from endosome to lysosome triggers the activation of viral proteins as pVI, responsible for fiber and penton base disassociation from the capsid, which causes endosomal membrane disintegration and virion release into the cytosol (Prchla et al. 1995). In the cytosol, the HAdV is translocated from microtube organization center (MTOC) to nuclear pore complex (NPC) for viral DNA import to the nucleus (Bailey, Crystal, and Leopold 2003). Once in the nucleus, the terminal protein (TP) attaches to nuclear matrix components for transcription, replication and, assembly of the mature virions. The adenoviruses progeny are released by cell lysis, triggered by the adenovirus death protein (ADP) (Fig.3) (Tollefson et al. 1996).

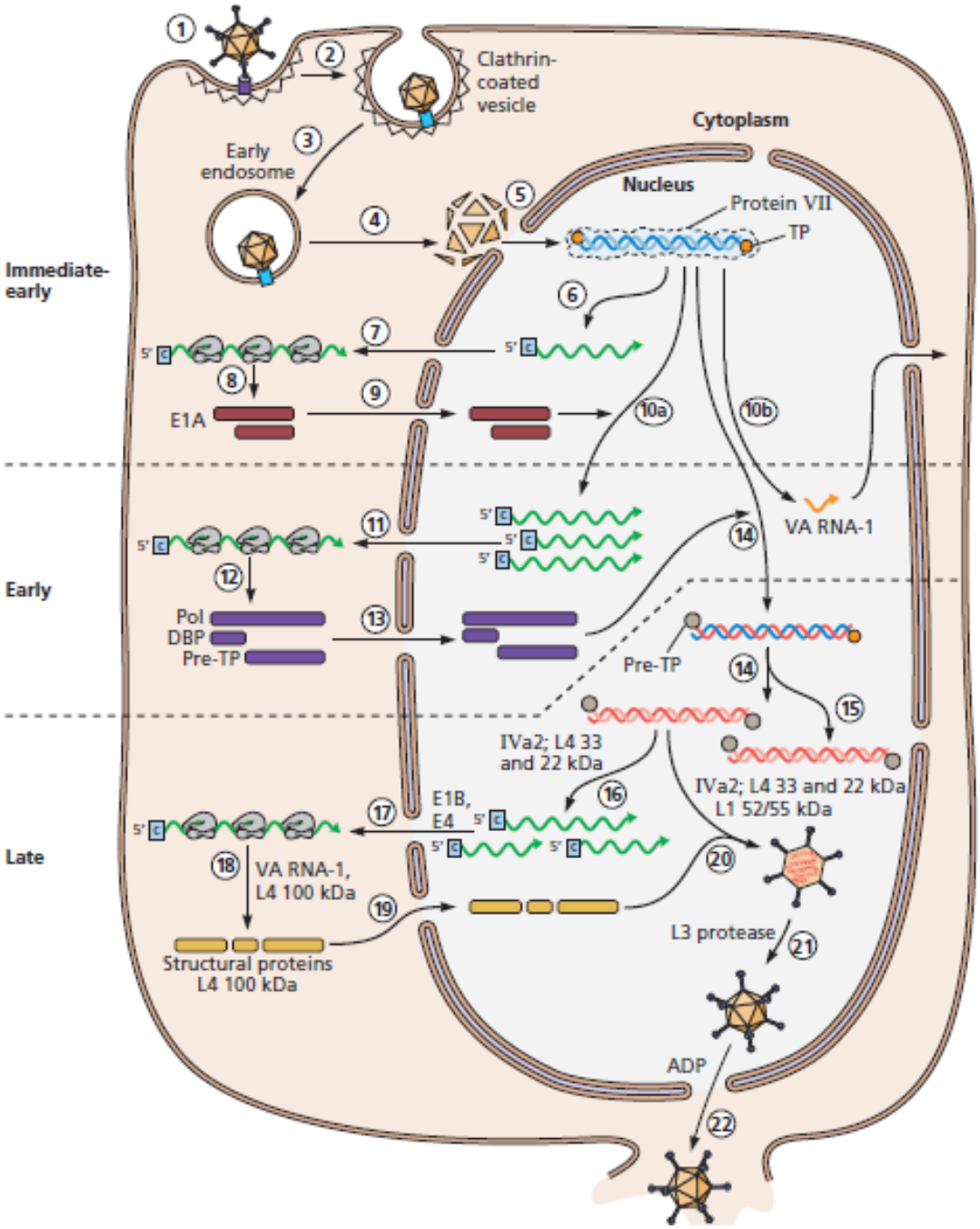

Figure 3- Global adenovirus infection phases: immediate-early, early and late (N. James MacLachlan and Edward J. Dubovi 2016)**.**

1.3. Viral genome organization and expression

The human adenovirus genome is about 26-45 kb in size and it is formed by linear double-stranded DNA molecules, which have inverted terminal repeat sequences (ITRs) at both ends containing the viral origins of replication (Rekosh et al. 1977; ZIFF and EVANS 1978; Davison, Benko, and Harrach 2003). Both 5' termini are covalently attached to a terminal protein (TP) (Hoeben and Uil 2013). The genome is transcribed by the host RNA polymerase II in three phases: early (E), intermediate and late (L) from 8 transcription units: early (E) (E1A, E1B, E2, E3, E4), intermediate (pIX, pIVa2) and late

(L) units (MLTU). The Major Late Transcription Unit (MLTU) process five transcripts (L1, L2, L3, L4, and L5). Additionally, VA RNAs are transcribed by RNA polymerase III (Fig.4) (Russell 2007; Shenk 1996; Schaack et al. 1991). The mentioned early genes are transcribed from the promotor MLP, as well as, the late genes (Russell 2007).

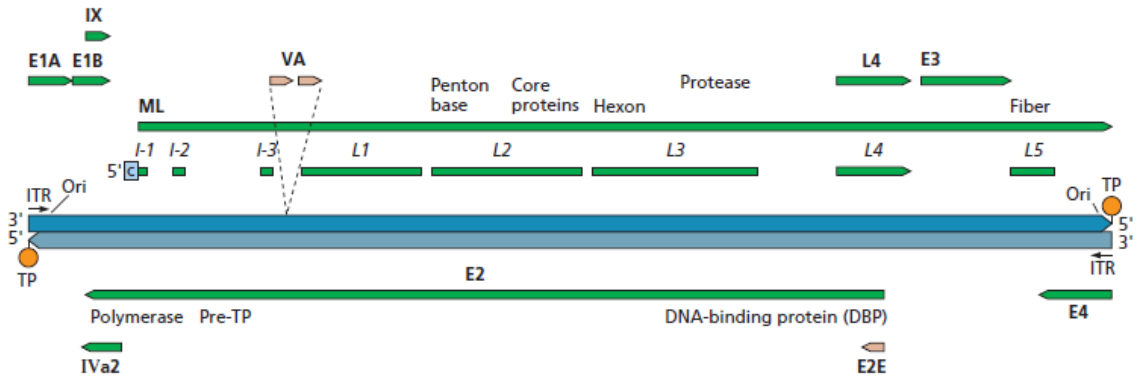

Figure 4- Adenovirus genome organization. *The genome is represented from the 5' end of the rightward strand. Arrows represent the organization and transcription direction of early (E1A, E1B, E2, E3, E4), intermediate (IX, IVa2) and late (L1-L5, ML) transcription units on both DNA strands. The genome is transcribed primarily by RNA polymerase II. In addition, two virus-associated RNA (VA-RNA) are transcribed by RNA polymerase III. ITR, inverted terminal repetition; Ori, the origin of replication; VA-RNA-Virus-associated RNA* (Flint 1994).

The early phase corresponds to the first phase of the adenovirus infectious cycle. This phase englobes the virus entry into the host cell, the viral genome passage to the nucleus and, the selective transcription and translation of early genes. The early events are important for cell functions modulation, to support the viral DNA replication and the production of the late genes (Russell 2007). The E-genes have distinct functions, E1 is involved in regulation and virus replication; E2 in replication; E3 in modulation and escape of the host immune system and E4 in regulation of DNA replication, mRNA transport and apoptosis (Hay et al. 1995; Täuber and Dobner 2001; Russell 2007). In the early phase, six components (E1A, E1B, E2A, E2B, E3 and E4) origin multiple mRNAs by differential splicing and alternative start codon usage (Berk et al. 1979). The adenovirus genome transcription starts with the E1A gene products expression, inducing the transcription of others regions in a synchronized way (Binger and Flint 1984). E1A encodes two major proteins, E1A-289R (or 13S) and E1A-243R (or 12S) that modulate cellular processes to enhance cellular susceptibility to the viral replication, such as regulation of NF-κB and the tumor suppressor p53 protein. E1B encodes two proteins, 19K and 55K involved in virus lytic cycle, viral replication and cellular apoptosis block (Zhao and Liao 2003).

The E2 gene products are subdivided into E2A (DBP) and E2B (pTP and Pol) (Täuber and Dobner 2001). The DNA binding protein (DBP), pre-terminal protein (pTP) and DNA polymerase (Pol) are involved in the viral DNA replication and subsequently, in the late genes transcription mediated by interaction with cellular factors. DBP works as a protein primer for replication initiation and the AdV Pol is associated with 5'- 3' polymerase activity and an intrinsic 3'-5' proofreading exonuclease activity (Liu,

Naismith, and Hay 2003). The DBP protein has a high affinity to ssDNA independent of ATP. This interaction destabilizes the helix structure of DNA and, stimulates the AdV Pol and NFI binding.

The E3-derived gene products modulate the host immune response against virus-infected cells and are non-essential for replication of the virus in tissue culture (Matthews and Russell 1998; Bennett et al. 1999). E3 proteins prevent the viral antigens transportation to the cell surface by MHC class I proteins and inhibit proapoptotic pathways (TNFα and FasL). The E4 region codifies a group of polypeptides (1-6/7) involved in transcriptional activation of heterologous promoters, preferential translation of viral mRNA instead of the host mRNA, viral DNA replication, host-cell protein synthesis shut-off and blockage of apoptotic pathways. The L-genes are transcribed after the E-genes, encoding structural proteins. The late transcription regions are transcribed from a common major late promoter (MLP) originating the major late transcript unit (MLTU) (Young 2003). After MLTU processing at 5 (or 6) different polyadenylation sites and differential splicing, forms the L1-L5 (or L6) transcripts. In the late phase of the cell cycle, the mRNAs are translated and carried to the nucleus. The late proteins are essentially viral structural components (core and capsid proteins) and 'helper' proteins (52/55K, 23K protease, 100K and 33K) important for correct assembly, encapsidation and maturation of virus particles in the nucleus (Table 1) (Molin et al. 2002). The most important structural proteins are the penton base (pIII), hexon (pII) and fiber (pIV), codified by L2, L3 and L5 region, respectively (Fig.5). The transcription and the expression of the genes are tightly synchronized, lasting the early phase 6-8h and the late phase 4-6h, resulting in about 50 polypeptides (Kay, Glorioso, and Naldini 2001; Gonçalves and de Vries 2006).

Table 1- Adenovirus structural proteins, molecular mass, localization, and functions (Bateman et al. 2017)**.**

Protein	**Molecular mass (kDa)**	**Localization**	**Function**
II	108	Hexon monomer	Structural
III	63	Penton base	Penetration
IIIa	65	Associated with the penton base	Penetration
IV	62	Fiber	Receptor binding
V	41	Core: associated to DNA and to the penton base	Histone-like
VI	27	Peptide associated with the hexon	Assembly; stabilization
VII	22	Core	Histone-like
VIII	25	Peptide associated with the hexon	Assembly; stabilization
IX	14	Peptide associated with the hexon	Assembly; stabilization
X (μ)	9	Core	----------
TP	77	Genome	DNA replication

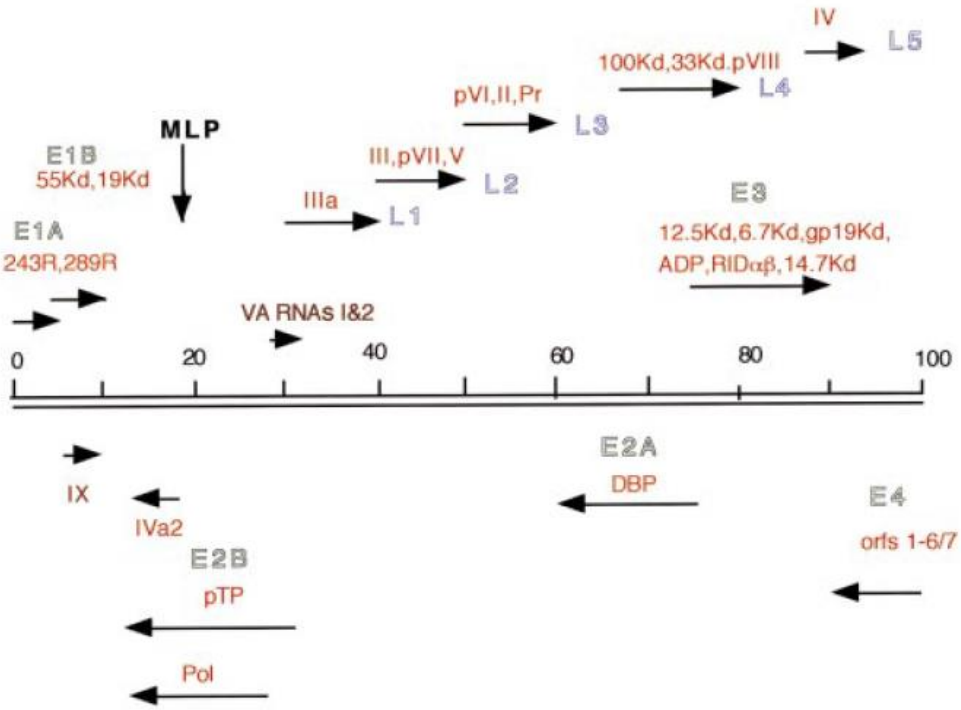

Figure 5- General overview of adenovirus transcription. *Arrows indicate the direction of transcription. MLP, Major late promoter* (Shenk 1996).

Overall, the viruses infectious cycle contemplates the virus entry in host cells, multiplication, and development of new virions. In the nucleus, the viral assembly process (e.g. replication) starts about 8 hours after infection, resulting in around 10^4 to 10^5 new particles production per cell. After 24 to 72h post-infection, the cell releases new virions.

1.4. Adenovirus impact and relevance

The adenoviruses worldwide problematic is related to water contamination and high survival to the water treatment (Jiang 2006). In recreational water, the annual risk of infection is around 1/1000 for a single exposure (World Health Organization 2005). HAdV has been considered as an index of enteric viruses due to the frequent occurrence in water sources and public health implications (Rattanakul, Oguma, and Takizawa 2015). Additionally, food environments are a major source of viral transmission to humans because of the food market fast globalization and changes in food consumption habits (Rodríguez-Lázaro et al. 2012). The frequent adenoviruses hexon evolution and variation within serotypes in type and extent of mutation cause the adenoviruses high plasticity. This plasticity may give rise to new adenoviruses strains and consequently to identification difficulties by serological methods. This virus is considered to be an emerging pathogen because (i) some incidences of adenoviruses' infection is growing; (ii) new infections have arisen from adenoviruses evolution, and (iii) infections have spread to previously unaffected areas (Marylynn V. Yates et al. 2006).

The particular viruses' mechanisms is useful to several applications, such as in life sciences and medicine (Doerfler W. 1996). For example, viruses can be used as vectors to introduce genes into cells or directly in the genome for function studies and to treat diseases by a genetically modified virus that exclusively reproduce in cancer cells (Jefferson, Cadet, and Hielscher 2015). Also, viruses can be regarded as organic nanoparticles for delivery antibodies, microRNA, etc (Fischlechner and Donath 2007). In addition to answering to a public health problem, the adenovirus inactivation also has applicability in numerous treatments focusing on vaccination.

Adenovirus disinfection methods

According to Environmental Protection Agency from US, for drinking water disinfection treatment it is recommended a removal of 99,99% (4 $\log_{10}$ PFU/mL) of enteric virus (US EPA 2018). However, conventional wastewater treatment systems are unable to provide virus-free effluents, removing only about 50-90% of enteric virus (Cloete, Silva, and Nel 1998). Previously precautions as good sanitary utilities and proper hygiene habits are very important to stop the progression of the viruses at early stages.

The conventional disinfection treatments include homogeneous chemical disinfection (e.g. ozone, chloride, chloride dioxide) and UV irradiation (Sigstam 2014). The majority of the inactivation mechanisms focused on genome damage, but protein damage may also contribute to viral inactivation. Chloride causes several protein alterations (e.g. oxidation, carbonylation, chlorination), however, UV irradiation focuses on genome damage, but proteins also can be affected (Hirneisen et al. 2010).

In addition to the conventional methods, the gamma radiation is a simple and effective disinfection method. The gamma rays have no mass and no charge providing a significant penetrating ability in liquid and solid material, being used to sterilize medical instruments, preserve food, and disinfect biological products. The radioisotopes involved in gamma radiation processes are cobalt-60 (60-Co) and caesium 137 (137-Cs). The better penetrating power and availability of cobalt-60 makes it more commonly used in industrial processing. Cobalt-60 radioisotopes decay to become non-radioactive nickel with an emission of a low and two high energy gamma rays, 0.318 MeV and, 1.17 and 1.33 MeV, respectively. The delivered dose in the target is relatively uniform, although the treatment efficiency can be influenced by protective agents of irradiation (Hansen JM 2001; Lambert PA 2004). The absorbed radiation dose is measured in the International System of Units as the Gray (Gy) or the kiloGray (kGy). One Gray equals the absorption of one joule of energy per kilogram (J/kg) (Olson 1995).

The gamma radiation mechanism of virus inactivation could be through two ways, direct and indirect. The direct inactivation occurs by radiolytic cleavage or crosslinking of critical targets (e.g. nucleic acids, proteins), and the indirect effects results of radiolytic cleavage of water in -OH, and, O2 in ozone (O) (Ohshima et al. 1996; Lomax, Folkes, and O'Neill 2013). The hydroxyl radicals are highly reactive causing oxidation, reduction, and the breakdown of C-C bonds of other molecules, including DNA. In addition to DNA interaction, the hydroxyl radicals and ozone also can interact with proteins. The major mechanism of virus inactivation by gamma radiation, via direct and indirect mechanisms, is believed to be the nucleic acids damage (Summers and Szybalski

1967). This preferential damage of nucleic acid rather than proteins is due to their G values. Nucleic acids have large G values compared to proteins or lipids, therefore, a large number of radiolytic species produced per 100 eV of absorbed energy, resulting in inactivated virus able to elicit immune responses (Skowron et al. 2014).

Irradiation treatment has been reported as a powerful tool to inactivate human pathogenic microorganisms in water, wastewater and sludge, even as in food and medical products while retaining nutrients and products characteristics (Madureira et al. 2017). The inactivation of microorganisms by ionizing radiation depends on the type of energy, dose rate and absorbed dose (Melo et al. 2008). Also, the response to ionizing radiation by the microorganisms could be influenced for several factors, for instance, the constitution of the irradiation medium (e.g., presence of protectors, sensitizers), irradiation atmosphere (e.g., air, N_2, vacuum), temperature, water content of the cell, age of the microorganisms and dose rate (Madureira et al. 2017). The target sensitivity to irradiation increases in aerobic environments owed to oxygen presence, also with water content, temperature elevation and presence of phosphate compounds (Hewitt and Leelawardana 2014).

Generally, the radio-sensitivity is proportional to the organism size and complexity. For example, a large organism (bacteria and fungi) is more sensitive to radiation compared to a smaller one (viruses). In viruses, large genomes may be more sensitive than small genomes within each single or double-stranded group. Although the single-stranded virus is smaller and simpler, also is radio-sensitivity homogenous thus, more sensitive to irradiation in comparison with the double-stranded virus. For double-stranded DNA effective break is required a simultaneous base-pair damage or inactivation of a segment of DNA critical for the virus (Farkas J 2007; Gazsó LG 2005). Additionally, alterations by ionizing radiation in proteins essential for host attachment and DNA injection into the host are responsible for decreases of viral infectivity.

The major lesions induced by ionizing radiation in nucleic acids are chemical alterations of deoxyribose sugar and, purine and pyrimidine bases (Hewitt and Leelawardana 2014). On the other hand, several organic and inorganic chemicals are able to act as radical ‘scavengers’ and protect from irradiation effects. Examples of protective components are proteins, carbohydrates, alcohols and sulfhydryl containing compounds. On the opposite side, there are nitrites, nitrates and quinones (Rayman, M M. 1958). The presence of scavengers in solution react with hydroxyl radicals and ozone preventing their capacity to act on viral proteins and nucleic acids (Hansen JM 2001).

Gamma radiation has been described as an effective technology to eliminate insects, fungi and bacteria without risks to the environment and human health. Although, the capacity of gamma radiation to eliminate viruses is less understood.

Enteric viruses like HAdV has a significant role in the environmental contamination, and the current methods of wastewater treatments do not always effectively remove these organisms. Understanding the behaviour of HAdV-5 under alternative disinfection treatments, as gamma radiation is fundamental for application of this methodology in wastewater treatment.

Objective

The main goal of this thesis project is to study the adenoviruses' inactivation mechanisms by gamma radiation treatment, focusing on the effects on genome and capsid proteins. The effects on adenovirus components and infection capacity were evaluated in PBS and wastewater substrates to mimic potential scenarios where ionizing radiation could be used as a disinfection tool for human enteric viruses. The insights on the inactivation of enteric virus via gamma radiation aim to contribute to develop alternative water disinfection process or complement the traditional treatments.

This project addresses ONU sustainable development goals, namely ensure availability and sustainable management of water and sanitation for all. Specifically, the ionizing radiation technology, such gamma radiation, could be applied to improve wastewater treatment and preserving water quality ("Agenda 2030 | ONU Brasil" 2018).

Material and methods

Material and methods

2.1. Viral stocks production and cell lines

Human adenovirus type 5 (HAdV-5; ATCC VR-1516) was propagated in human lung carcinoma cells A549 (ATCC CCL-185). A549 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; Hyclone, Gel Life Sciences, Logan, Utah) supplemented with 10% fetal bovine serum (FBS) (heat inactivated; Hyclone, Gel Life Sciences, Logan, Utah), 100 units/mL penicillin, 100µg/mL streptomycin, 1 mM sodium pyruvate and 10 mM HEPES buffer at 37ºC under a 5% CO_2 atmosphere. To prepare HAdV-5 stocks, confluent A549 cells were infected with inoculum containing 10^7 PFU/mL using a multiplicity of infection (MOI) of 0.1. After 7 days post-infection, viruses were harvested by three freeze-thaw cycles and centrifugation at 3,000 rpm, (Beckman J2-21M, rotor J20-1) for 30 min at 18ºC. The resulting supernatants were stored at -80ºC. The titre of virus stock was estimated by plaque assay.

2.2. Samples preparation

HAdV-5 samples (2 mL) were prepared from HAdV-5 virus stock (10^{10} PFU/mL) suspended in phosphate-buffered saline (PBS) and wastewater to achieve a final concentration of approximately 10^8 PFU/mL. Wastewater (COD 57 mg O_2/liter) was collected before tertiary treatment from a municipal wastewater treatment plant (Constância, Portugal).

HAdV-5 DNA samples were obtained after viral DNA extraction using PureLink Viral RNA/DNA kit (Invitrogen, Carlsbad, CA, USA) that was eluted in 50 µL of RNase/DNase-free water supplied in the extraction kit.

2.3. Irradiation process

The samples were irradiated in a Cobalt-60 experimental chamber (Precisa 22; Graviner Manufacturing Company Ltd., United Kingdom; 1971) at room temperature with an activity of 165 TBq (4.45 kCi) and a dose rate of 1.2 kGy/h located at Campus Tecnológico e Nuclear (Bobadela, Portugal). The dose rate was determined by Fricke dosimetry and the absorbed doses were measured by routine dosimeters (batch X; Amber Perspex Harwell, London) with nominal uncertainty limits of about 5% (Atomic and Agency, n.d.).

Samples were irradiated at gamma radiation doses ranging from 4 up to 25 kGy (Table 2). A gamma radiation dose range with lower doses (1.5 to 25 kGy) were also tested for virus suspensions in PBS at an approximate initial concentration of 10^9 PFU/mL. All irradiations were performed in triplicate. Non-irradiated samples (0 kGy) followed all the assays.

Table 2- Gamma radiation real dose measured by routine dosimeters. *Average values of Dose Rate (DR) and Dose are presented; For doses <10 kGy and >10 kGy were considered the absorbance at 603nm and 651nm, respectively.*

Exposure Time (h)	DR (kGy/h)	Average Dose ± standard error (kGy)
1:15	1.2	1.5 ± 0.3
2:30	1.2	3.1 ± 0.1
3:30	1.2	4.4 ± 0.4
12:30	1.2	15.3 ± 0.2
20:30	1.2	25.0 ± 0.2

2.4. Virus titration

The HAdV-5 viral titer of non-irradiated and irradiated samples was determined by plaque assay in A549 cells. The cells were seeded and incubated into 60-mm plates at 37°C and 5% CO_2. The cellular monolayers with 60-80% of confluence were infected with 300 µL of 10-fold serial dilutions of non-treated and 4 kGy treated samples in duplicates. In the case of 15 kGy and 25 kGy treatment, performed non-diluted sample inoculum of 500 µL was used. Duplicates were made for each sample. The cells were incubated for 1 hour at 37°C and 5% CO2, with mild agitation every 15 min. After the incubation, the inoculum was removed, and the cellular monolayer was overlaid with 3 mL of 1x MEM combined with 0.5% agarose. After 3 and 7 days, a 1.5 mL overlay of 1x MEM with 0.5% agarose was added. The third overlay also contains 1% of a neutral red solution (Sigma, St. Louis, MO, USA). Viral plaques were counted 8 to 24h after the last overlay and the viral titer was determined by the following equation:

$$\text{PFU/mL} = \frac{plaques\ counted}{volume\ of\ infection\ (mL) \times dilution}$$

Virus titer was expressed in decimal logarithm of PFU per millilitre (Log PFU/mL).

2.5. Adenovirus DNA extraction and Long PCR

As mentioned before, viral genomic DNA was extracted from HAdV-5 suspensions (non-irradiated and irradiated samples) using the PureLink Viral RNA/DNA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Briefly, a total volume of 200 µL of HAdV-5 (non- and irradiated) suspensions was used per DNA extraction. The purified nucleic acids were eluted in 50 µL of RNase/DNase-free water and stored at -20ºC until long PCR analysis was undertaken. Additionally, an eluted volume of 50 µL was firstly irradiated at same dose range (4 to 25 kGy) and then submitted to the PCR analysis. The Adenovirus Long PCR was performed using My Taq

Red reaction buffer (Bioline, London, United Kingdom) and My Taq HS DNA Pol (Bioline, London, United Kingdom). The primers used to flank a 1- and 10-kb fragment (Fig. 6-7), using for both the forward primer AD2 For178 5'- CGG CGG TAT CCT GCC CCT CC-3' were combined with the reverse AD2 Rev189 5'- CGT AGG TGC CAC CGT GGG GTT TCT AAA C-3' and AD2 Rev278 5'- CGC TCT GCC TCT CCA CTG GTC ATT CAG TC -3', respectively (Table 3). For long PCR, 5 µL of extraction product was added to 20 µL of a reaction mixture containing 1X My Taq Red reaction buffer (Bioline, London, UK), 0.3 µM of each primer and 2U of My Taq DNA polymerase (Bioline, London, UK). The Long PCR conditions were as follows: initial temperature of 95ºC for 90s followed by 15 cycles of 94ºC for 15s, 65ºC for 10 min, followed by 1 cycle of 72ºC for 10 min. The amplifications were performed in a Mastercycler gradient thermocycler (Eppendorf). PCR products were analysed on 2% agarose gel (stained with gel red; Biotium, Hayward**,** CA, USA) electrophoresis at 100V for 90 min and was visualized under UV light. The molecular weight marker 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA) was used to estimate the amplification product size. The PCR technique was used for genome detection, however, for infectious and non-infectious viral particles discrimination other complementary methods were used.

Table 3- Primers for long-range PCR, orientation, bases, sequence, GC content, temperature of melting, position, fragment size and genes (Rodríguez, Bounty, and Linden 2013).

Name	Orientation	Bases	Sequence	GC content (%)	T_m (ºC)	Position	Frag. size (bp)	Genes
AD2 For178	Forward	20	5'- CGG CGG TAT CCT GCC CCT CC-3'	75	65.2	17822-17842		
AD2 Rev189	Reverse	28	5'- CGT AGG TGC CAC CGT GGG GTT TCT AAA C-3'	57.1	64.3	18969-18996	1174	pVI
AD2 Rev278	Reverse	29	5'- CGC TCT GCC TCT CCA CTG GTC ATT CAG TC -3'	58.6	65	27893-27929	10107	pVI; hexon; protease; DBP; 100K; 33K; 22K; pVIII

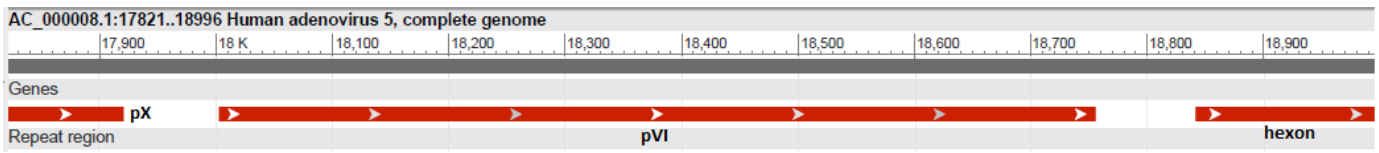

Figure 6- 1-kb fragment codifying region. *The fragment contents minor protein (pVI) gene and, a partial region of hexon (pII) and pX genes* (Brister et al. 2015).

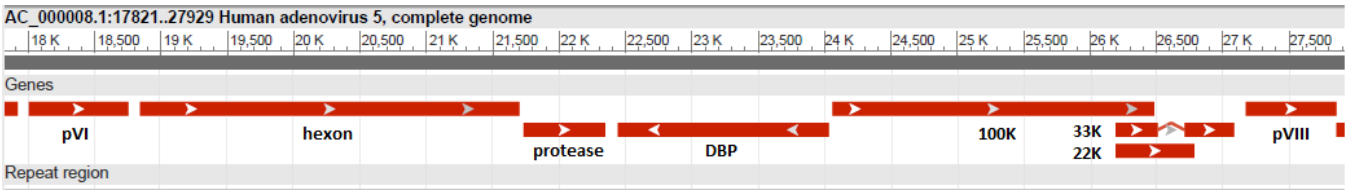

Figure 7- 10-kb fragment codifying region. *The fragment contents the genes of minor coat: pVIII; core: pV; structural: hexon (pII); helper: protease (23K),100K, 33K, 22K; and DNA-binding: DBP proteins (Brister et al. 2015) .*

2.6. Analysis of viral proteins by SDS-PAGE

The analysis was performed using a stacking and resolving gels with polyacrylamide concentrations of 4 and 12.5%, respectively. The polyacrylamide resolving gel is constituted for 12.5% acrylamide/ BIS-acrylamide (Sigma, St. Louis, MO, USA), 0.1% TEMED (Sigma, St. Louis, MO, USA), 0.1% ammonium persulfate, 0.4 M tris (pH 8.8) and 0.1% SDS. In addition, the stacking gel contains 4% acrylamide/BIS-acrylamide, 0.1% TEMED, 0.1% ammonium persulfate, 0.13M tris (pH 6.8) and 0.1% SDS). Gels were poured into pre-made gel chambers. Combs was immediately added, and the gel was left to polymerize. After polymerization, combs were removed, and the polyacrylamide gel was inserted in the Mini-PROTEAN Tetra Cell (BioRad, Germany) and filled with running buffer (0.025M tris base (pH 6.8), 0.2M glycine and 0.1% SDS). Ten microliters of HAdV-5 samples (either gamma radiation treated or untreated) were boiled in an equal amount of 1X laemmli buffer (60 mM Tris HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) during 5 minutes. After that, samples were loaded onto the polyacrylamide gels with the molecular weight marker NZYBlue Protein Marker (Nzytech, Lisboa, Portugal) and ran at a constant current of 100V for 90 min. Gels were incubated overnight with BlueSafe staining (Nzytech, Lisboa, Portugal) for proteins visualization.

2.7. Western- blotting

Subsequently to proteins separation by SDS-PAGE, proteins were transferred onto a nitrocellulose membrane using the following method. Transfer cassettes contained the following layers: a sponge, Mini Trans-Blot filter paper (BioRad, Germany), 0.45µm nitrocellulose membrane (BioRad, Germany), SDS-PAGE gel, Mini Trans-Blot filter paper and a sponge (all equipment was pre-immersed in transfer buffer containing 20% v/v methanol, 192 mM glycine and 25 mM tris). The transfer cassette was then placed in the transfer tank Mini- PROTEAN Tetra cell (BioRad, Germany), filled with transfer buffer and ran for 90 minutes at a constant amperage of 400 mA. After the transferring, nitrocellulose membranes were washed in PBST (1% Tween-20 in PBS) and saturated with blocking buffer (5% skim milk in PBST) overnight at 4ºC to avoid nonspecific antibody binding and to reduce the background. For HAdV-5 viral proteins samples, the blot was probed with goat anti-adenovirus polyclonal IgG antibody (0151-9004, Bio Rad) at a dilution of 1:100 in blocking buffer during 1h at room temperature with constant agitation. Following antibody incubation, membranes were washed three times in PBST for 10 min under agitation. Then the membrane was incubated during 1h with the rabbit anti-goat IgG secondary antibody (STAR 122P, Bio Rad) at a dilution of 1:1000 in

blocking buffer. Membranes were subsequently washed in PBST for 10 min with gentle agitation to remove the unbound stain. Protein sizes were compared to NZYBlue Protein Marker (Nzytech, Lisboa, Portugal). Protein detection by colorimetric detection of signals was performed by western amplification module (170-8230, Bio Rad) and Opti-4CN substrate kit (1708235, Bio Rad) according to manufacturer's instructions. The transfer efficiency of proteins onto membranes during western-blotting was confirmed by reversible staining with BlueSafe staining (Nzytech, Lisboa, Portugal) for at least 30 min.

2.8. ELISA

Enzyme-linked immunosorbent assay (ELISA) was realized using the Ridascreen adenovirus kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions. This assay contains monoclonal antibodies prepared against adenovirus-specific hexon proteins of the virus capsid. Non-irradiated and irradiated HAdV-5 samples in PBS and wastewater were analysed at an initial volume of 100 µL by the ELISA technique. Each sample was tested in duplicate. Absorbance was measured by a microplate reader, EZ Read 800 (Biochrom), at 405 nm, using 620 nm as a reference. The percentage of relative binding was inferred by $[S_i/S_{ni}]*100$. Where S_{ni}, is the value of non-irradiated sample and S_i the value measured after irradiation.

Results and Discussion

Results and Discussion

Effects of gamma radiation on adenovirus viral proteins

In order to study the influence of gamma radiation on Human Adenovirus 5 (HAdV-5) proteins, five initial doses were selected, 1.5, 3, 4, 15 and 25 kGy. At lower doses, 1.5 and 3 kGy, the gamma radiation effect on proteins indicated to be insufficient to degrade the HAdV-5 proteins (Fig.8), thereby, the sequential trials only consider the doses 4, 15 and 25 kGy. For the applied doses of 15 kGy and 25 kGy the proteins profile obtained by western-blotting was altered indicating that gamma radiation, at these doses, could degrade some HAdV-5 proteins (Fig.8).

To evaluate the substrate composition influence on degradation of viral proteins by gamma radiation, the virus suspensions were prepared in PBS and wastewater. The PBS was used since it is a neutral substrate similar to water and not cytotoxic to host cells. The wastewater substrate was used to mimic a potential scenario where ionizing radiation could be used as a disinfection tool for human enteric viruses (Pimenta et al. 2016). The distinct substrates could allow to evaluate the trends of gamma radiation inactivation response. Additionally, the susceptibility of the specific viral proteins to gamma radiation could be pointed out based on the applied methodology.

The western-blotting assay was performed to determine whether the proteins remain antigenic after gamma radiation treatments, using a polyclonal antibody to recognize adenovirus hexon and other proteins. At 1.5, 3 and 4 kGy gamma radiation doses, the visualized proteins are similar to the untreated sample (0 kGy dose). However, the increase of applied dose was related with a decrease of antibody-binding signals. As shown in Fig.8, at 15 and 25kGy, the structural proteins of HAdV-5 capsid, hexon (pII), fiber (pIV), penton (pIII) and minor protein pIIIa with a molecular mass of approximately 108, 62, 63 and 65 kDa were visualized by western-blotting, although with lower signal intensity compared with non-treated sample (0kGy). Results suggested that HAdV-5 structural proteins of capsid were less sensitive to gamma radiation and these proteins could have a role on the resistance to gamma radiation. These data demonstrate that viral proteins were degraded after gamma treatments with different sensitivity to irradiation. Also, for a lower initial concentration of virus (10^8 PFU/mL), the pIII and pIV proteins are visualized at 4 kGy for wastewater substrate (Fig.9). The trial outputs correlate with the previously determined D_{10} value for PBS and wastewater of 0.9 kGy and 1.3kGy, respectively (Pimenta et al. 2016). The wastewater substrate requires a higher dose to inactivate 90% (1 $\log_{10}$ reduction) of a population, demonstrating higher radioresistance and, consecutively higher proteins presence after gamma radiation when compared with the PBS substrate.

The organic matter was also an important factor. The chemical oxygen demand (COD) represents the measurement of the oxygen required to oxidize soluble and particulate organic matter in water. The PBS and wastewater substrate COD values are < 50 and 57 mg O_2/liter, respectively. The lower levels of COD for PBS substrate prevent scavenging and HAdV-5 protection from radiolysis. In PBS substrate the effect of gamma radiation on HAdV-5 proteins was more pronounced than the verified for wastewater

substrate (Fig.9). The substrate organic matter has a protective role from indirect effects of gamma radiation, acting as a scavenger in wastewater substrate.

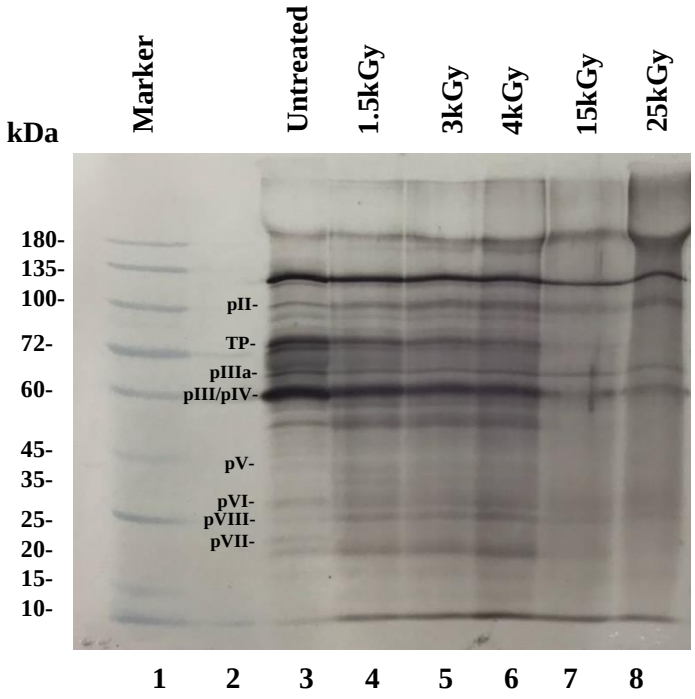

Figure 8- Western-blotting of proteins of HAdV-5 suspensions on PBS (10^9 PFU/mL) treated with 1.5, 3, 4, 15 and 25 kGy gamma radiation. *Untreated HAdV-5 proteins represent the 0 kGy treatment. Hexon (pII):108kDa; TP: 77kDa; pIIIa: 65kDa; pIII: 63kDa; pIV: 62kDa; pV: 41kDa; pVI: 27kDa; pVIII: 25kDa; pVII: 22kDa; pIX: 14kDa; pX: 9kDa.*

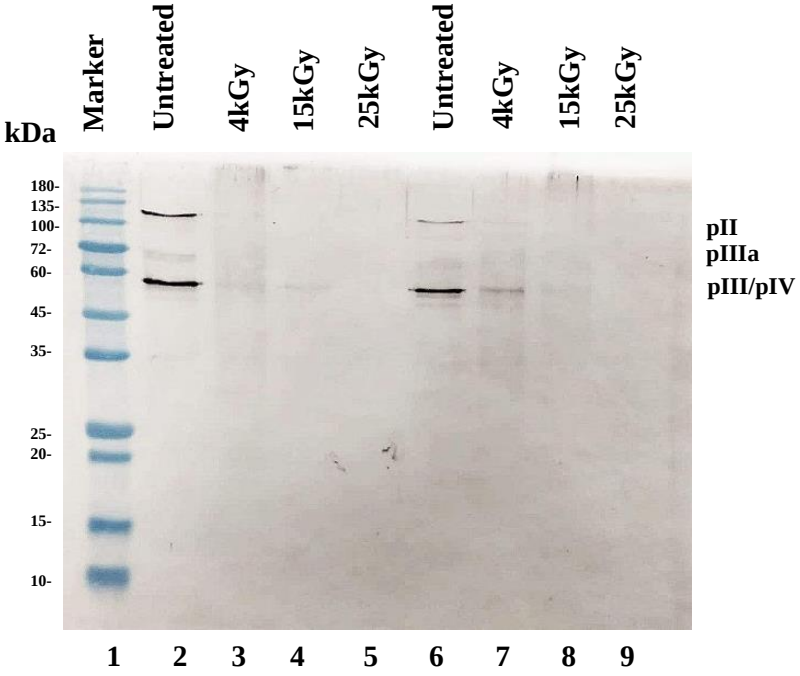

Figure 9- Western-blotting of proteins of HAdV-5 suspensions (10^8 PFU/mL) treated with 4, 15 and 25 kGy gamma radiation. *Untreated HAdV-5 proteins represent the 0 kGy treatment. (2-5) HAdV-5 suspended in PBS and (6-9) HAdV-5 suspended in wastewater.*

The viral capsid proteins hexon, penton and fiber proteins are mainly responsible for the human adenovirus complexity, due to its individual function and the organization in an intricate network (Keswick et al. 1985). The capsid proteins damage compromises the protection of viral genetic material and, subsequently the viral DNA replication and host infection (Bosshard et al. 2013).

The capsid antigenicity was also evaluated through the capacity of HAdV-5 samples bind to anti-hexon monoclonal antibodies by ELISA assay. The evaluation of hexon HAdV-5 proteins antigenicity was performed on virus suspended in PBS and wastewater substrates. The relative binding of the HAdV monoclonal antibody (MAbs) was calculated based on the obtained values for non-irradiated control samples, which were considered to correspond with 100% of binding HAdV MAbs.

Based on ELISA analysis, the irradiation damage on the viral capsid was influenced by the substrate. PBS substrate demonstrated to be more radiosensitizing than the wastewater substrate. The percentage of relative binding at 4kGy for PBS substrate was 17%, contrasting with 88% for wastewater (Fig.11). These results support the ones obtained for the same treatment dose in western-blotting assay, where it was also observed a protective effect to the viral hexon protein by wastewater components gamma radiation radiolysis (Fig.9). The recognition signals of HAdV-5 suspensions irradiated with higher applied doses (15 and 25 kGy) were not detected (negative result according to the manufacturer's instructions) for both substrates (Fig.10 and Fig.11).

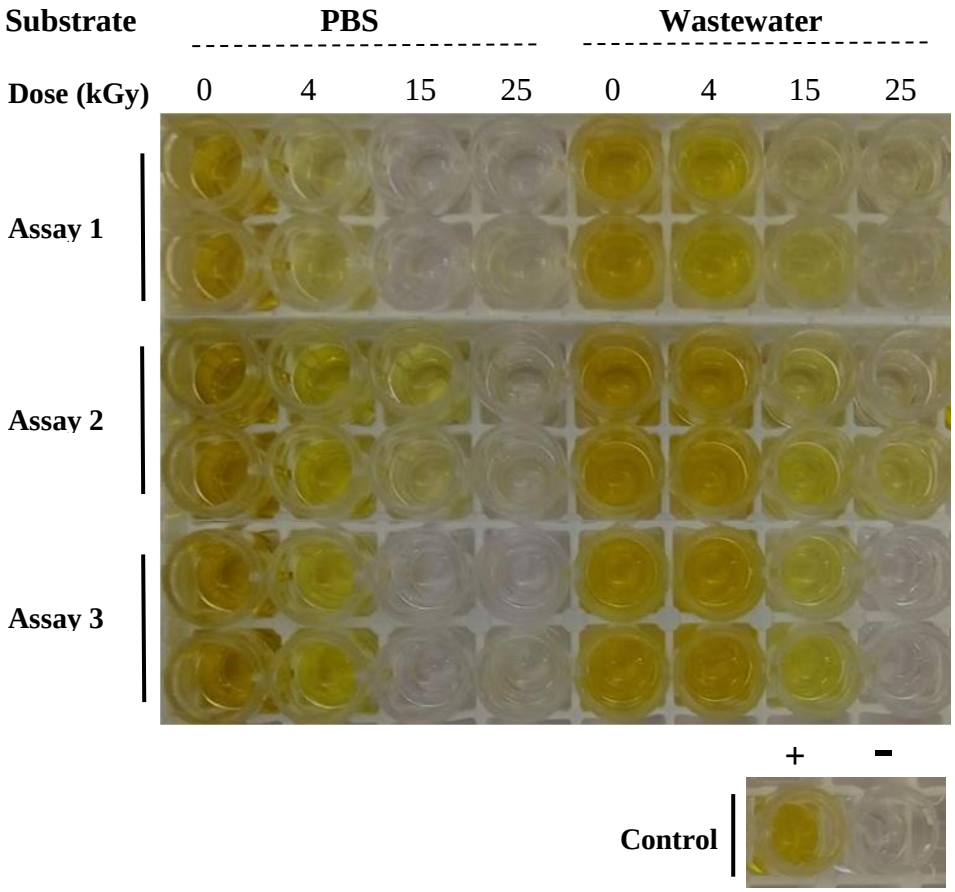

Figure 10- ELISA assay using Ridascreen adenovirus kit. *The assay was performed for PBS and wastewater substrates at 0 (untreated sample), 4, 15 and 25 kGy dose. Each assay was performed in duplicate. Positive (yellow) and negative (white) controls followed all assays. Absorbance was measured at 405 and 620 nm by a microplate-reader.*

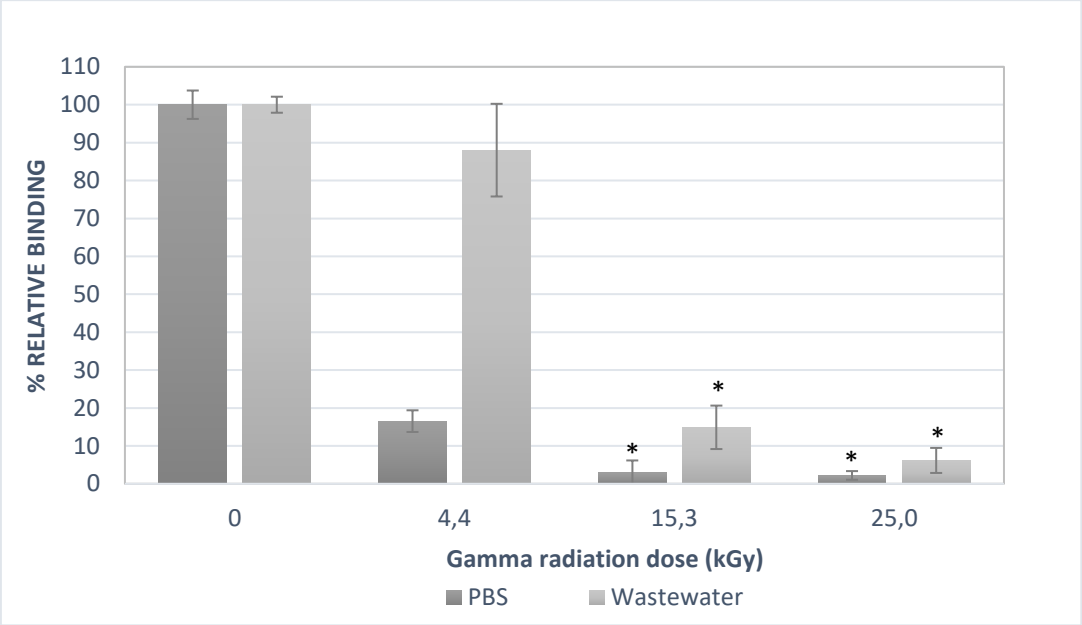

Figure 11- Qualitative detection by ELISA using a monoclonal antibody to the hexon antigen of human adenovirus types of HAdV-5 suspensions on PBS and wastewater that were untreated (0kGy control) and treated with different doses of gamma radiation (4 kGy, 15 kGy, and 25 kGy). *Bars represent the relative percentage to control HAdV-5 antibody binding. Error bars correspond to the standard errors about the mean values of the two replicates of three irradiation batches (n=6). *, negative result according to the manufactur's instructions.*

Effects of gamma radiation on adenovirus genome

To detect the damage on HAdV-5 DNA, sets of primers were used to analyse a region of the genome between 17.8 and 27.9 kb, about 10- in 35.9-kb of the total genome. Sets of primers amplify two distinct fragment sizes with about 1- and 10-kb. To evaluate the difference in the use of a 1- and 10-kb fragment size to detect HAdV-5 DNA damage, viruses were treated with gamma radiation at five doses (1.5, 3, 4, 15 and 25kGy) and viral DNA was extracted from each sample, followed by Long-PCR amplification.

The 1-kb fragment contains the pVI adenovirus gene, and the 10-kb the succeeding genes: pVI, hexon (pII), protease (23K), DBP, 100K, 33K, 22K and pVIII. All genes belong to the viral late region 3 and 4, responsible for capsid assembly and stabilization. The amplification products were analysed on 2% agarose electrophoresis gel. The 1- and 10-kb fragment showeds distinct susceptibility to gamma radiation dose. The 1-kb fragment was amplified in all HAdV-5 samples (non-treated 0 kGy and treated 1.5, 3, 4, 15, 25 kGy), nevertheless it was observed for the samples irradiated at 25 kGy a slight different fragment amplification size (Fig.12a). The HAdV-5 DNA is protected from radiation through the capsid. Also, the DNA damage and consequently non-amplification by PCR require a simultaneously double break of HAdV-5 DNA. For 10-kb amplification fragment, that represents about 1/3 of the total HAdV-5 genome, the minimum radiation dose required for non-PCR amplification was 4 kGy (Fig.12b). The increasing size of fragment puts in evidence further DNA damages at low radiation doses preventing DNA amplification.

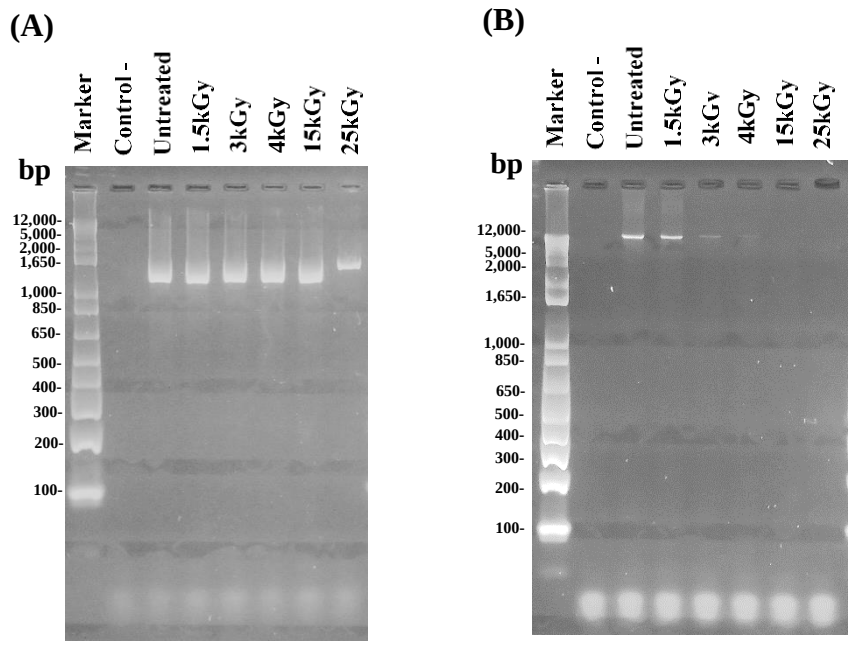

Figure 12- HAdV-5 samples suspended in PBS, irradiated at 1.5, 3, 4, 15 and 25 kGy and, after that the DNA was extracted and submitted to Long-PCR assays to amplify a (A) 1-kb and (B) 10-kb fragment of genome. *Untreated samples (0 kGy) and negative control (control -) followed all assays.*

The substrate role in HAdV-5 genome damage was tested for PBS and wastewater. The minimum gamma radiation dose for non-PCR amplification of the 1-kb fragment in both substrates was 15 kGy (Fig.13). Accordingly to these results, the HAdV-5 DNA damage suggested to be independent of the substrate considering the two substrates analysed PBS and wastewater.

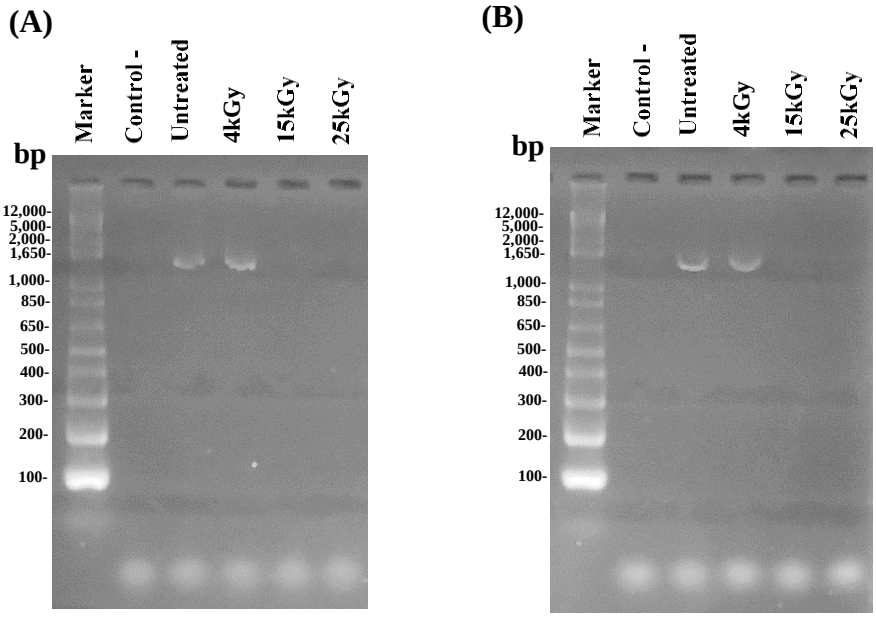

Figure 13- HAdV-5 samples suspended in (A) PBS and (B) wastewater were irradiated at 4kGy, 15kGy and 25 kGy and, after that the DNA was extracted and submitted to Long-PCR amplify 1-kb fragment of viral genome. *Untreated samples (0 kGy) and negative control (control -) followed all assays.*

Complementary studies of DNA damage resulting from gamma radiation were performed for HAdV-5 DNA extracted and after that irradiated at three gamma radiation doses (4, 15, 25 kGy). For the irradiated samples, the 1-kb fragment was only amplified for the irradiated at 4 kGy (Fig.14a), but the 10-kb fragment was not amplified for any irradiated sample (Fig.14b). The role of HAdV-5 capsid in DNA protection from gamma radiation point out to be crucial for the higher doses applied of 15 kGy and 25kGy, comparing the amplification results from capsid protected DNA (Fig.12a) and non-protected DNA (Fig.14a). Regarding the 10-kb amplification fragment the results were similar for the analysis of the viral DNA with (Fig.12b) and without (Fig.14b) the capsid.

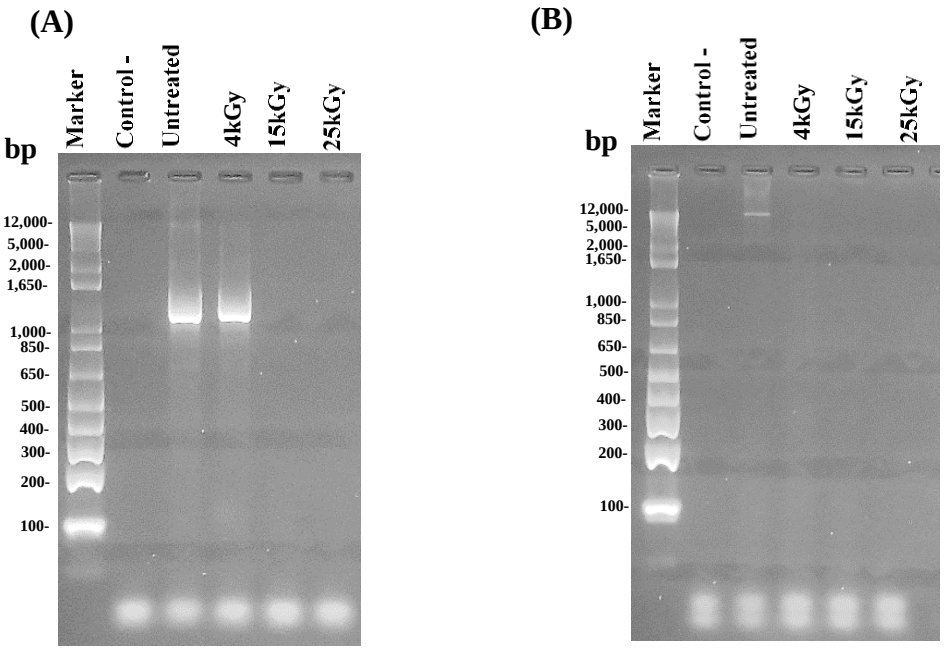

Figure 14- HAdV-5 DNA extracted and then irradiated at 4 kGy, 15 kGy and 25 kGy suspended in RNase/DNase-free water and submitted to Long-PCR to amplify (A) 1-kb and (B) 10-kb fragment. *Untreated samples (0 kGy) and negative control (control -) followed all assays.*

Effects of gamma radiation on adenovirus infectivity

Virus infectivity was analysed by plaque assay technique using A549 cell line. When a suspension of a microorganism is irradiated at different radiation doses, the number of surviving microorganisms after each dose may be used to construct survival curve, that is, a log variation of the surviving fractions in function of the absorbed radiation dose (kGy). Figure 16 shows the logarithmical target virus' titer reduction measured by plaque assay after irradiation at several gamma radiation doses for the two tested substrates, PBS and wastewater.

The viral $\log_{10}$ reduction for PBS and wastewater samples irradiated at 4 kGy was 6.5 and 5.0, respectively. At higher doses, 15 and 25kGy, HAdV-5 infection particles were not detected by the applied methodology (Fig.15). Therefore, supporting the results

obtained in the viral proteins analysis, the virus demonstrated to be more sensitive to gamma radiation when suspended in PBS than in wastewater.

The effect of HAdV-5 infection on A549 cells was visualized by an optical inverted microscope. The untreated (0kGy) PBS and wastewater samples showed cytopathic effect, through morphology alterations and diminution of cells number (Fig.17a,e). In the case of samples irradiated at 4 kGy doses, there was a visible reduction of cytopathic effect in both substrates (Fig.16b,f). The effect of HAdV-5 PBS and wastewater samples irradiated at 15 and 25kGy doses on cell line growth indicated to be similar to non-infected samples (negative control). For these doses was not visible a cytopathic effect (Fig.16d,h).

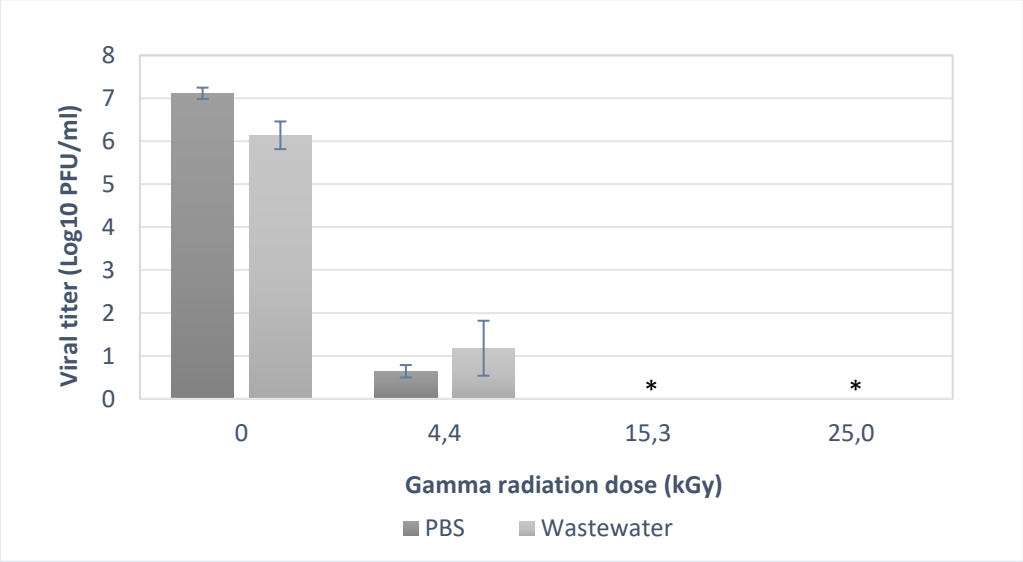

Figure 15- Survival curve of HAdV-5 suspended in different substrates (PBS and wastewater) and treated by gamma radiation (4 kGy, 15 kGy and 25 kGy). *Error bars correspond to the standard errors about the mean values (n=6). * <0.3 Log PFU/mL – detection limit of plaque assay.*

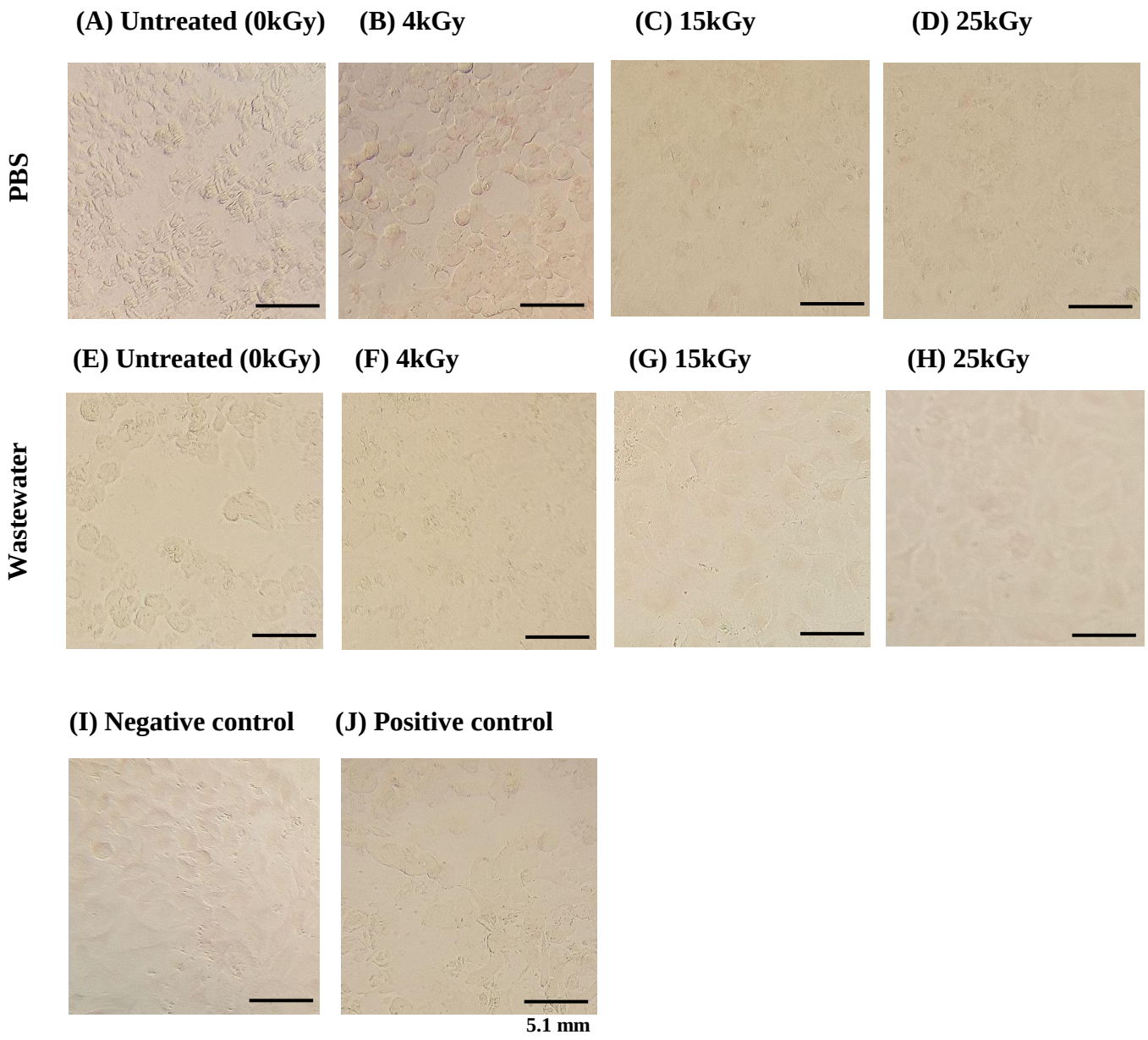

Figure 16- Visual effect of HAdV-5 samples on A549 cells. *(A-D) PBS and (E-H) wastewater samples non-irradiated 0 kGy, and irradiated at 4 kGy, 15 kGy and 25kGy. Non-infected cells (negative control, I) and cells infected with HAdV-5 stock (positive control, J) followed all the assays.*

Viral DNA damage and infectivity

The damage of HAdV-5 DNA was compared with the reduction in viral infectivity after gamma radiation treatment. In the case of 4 kGy treatment, the reduction of viral infectivity on PBS and wastewater substrates seems to be higher than the reduction/absence of DNA amplification of 1-kb fragment (Fig.13). The plaque assay was able to detect approximately 1 log PFU/mL viral titer on PBS and wastewater HAdV-5 samples irradiated at 4 kGy.

When a dose of 15 and 25kGy was applied to HAdV-5 suspended in PBS or wastewater, no amplification of viral DNA (Fig.13), agreeing with the results of plaque assay (viral titer < 0,3 $\log_{10}$ PFU/ml) (Fig.16).

Viral proteins damage and infectivity

Viral proteins are responsible for a critical part of the viral infection. For the HAdV-5 proteins profile it was evaluated its abundance/degradation trend with irradiation doses and antigenicity. The proteins abundance decrease observed for viral samples on PBS and wastewater irradiated at 4 kGy correlates with loss of viral infectivity (log reductions of 6.5 and 5.0 for PBS and wastewater, respectively) (Fig.15). At higher doses, 15 kGy and 25 kGy, the antigenicity of viral proteins was lower, as well as, the detected infectious virus (< log 0.3 PFU/mL).

Conclusion: Adenoviruses′ inactivation mechanisms

In the previous studies, it is thought that the main virus's inactivation mechanism by gamma radiation is the genetic material damage (Summers and Szybalski 1967). Gamma rays have direct and indirect effects on target molecules. They can directly 'hit' the genome or indirectly interfere with genome via free oxygen radicals formed by water radiolysis, which originates nucleotide degradation, single- or double-strand breaks and cross-linkage breaks (Lomax, Folkes, and O'Neill 2013). The amount of HAdV-5 amplified genes decreased as the irradiation dose increased is consistent with this.

Additionally to DNA degradation, the gamma radiation also damaged the viral proteins. The viral proteins abundance and antigenicity decreased as the gamma radiation dose increased. The gamma radiation damage included viral capsid disruption and physically distorting the virion conformation. Examples of gamma radiation targets are covalent and noncovalent bonds (van der Waals forces, ionic and hydrogen bonds), as well as, hydrophobic interactions responsible for proteins structure (Feng et al. 2011). Interestingly, for doses between 1.5 kGy and 4 kGy was observed intermediate products <35 kDa produced by gamma radiation (Fig.8). Proteins fragments and small peptides <10 kDa were too small to be resolved by SDS-PAGE. Nevertheless, others viral proteins still react with adenovirus polyclonal antibody at 4 kGy, suggesting the maintenance of the primary amino acid sequence.

The viral HAdV-5 DNA region of 1-kb was amplified at higher doses, 15 kGy and 25kGy, contrasting with a lower abundance of antigenic proteins for the same gamma radiation doses. These outputs support the fact that HAdV-5 capsid act as a shield for DNA protection. Although, the HAdV-5 DNA damage from irradiation also could not be included in the region that was amplified by Long-PCR. The 1-kb fragment was amplified after 4 kGy, 15 kGy and 25kGy irradiation treatments, while the 10-kb fragment not, suggesting its adequacy to assess DNA damage.

Despite the 1-kb HAdV-5 DNA integrity maintenance after virus irradiation at 15 kGy and 25 kGy (Fig.12a), the proteins antigenicity signals (Fig.8) and viral infectivity

decreases. It can be hypothesized that firstly, the gamma radiation targets the HAdV-5 proteins and subsequently breaks the viral DNA in fragment sizes higher than 1-kb. For samples treated at 4 kGy gamma radiation dose, the HAdV-5 proteins were visualized but the 10-kb fragment was not be amplified and, the viral infectivity is significantly reduced. The results suggested that HAdV-5 DNA degradation is the main factor for viral infectivity loss and HAdV-5 inactivation by gamma radiation.

The viral infectivity reduction and proteins preservation as the HAdV-5 DNA damage increase allows the host to produce antibodies to fight HAdV-5 infections. The results are hopeful for vaccines based on adenovirus.

On the other hand, even without the detection of 10-kb fragment amplification, the HAdV-5 samples irradiated at 4 kGy presented infectious potential for A549 cells. A viral HAdV-5 particle produces around 10^4 to 10^5 new virions per cell. The PCR methodologies may not detect the DNA of a viral HAdV-5 particle, but this one can posteriorly infect and produce a raised number of new virions. These data must be considered for new methodologies application to evaluate virucidal activity.

The substrate composition influence was tested on viral inactivation by gamma radiation. The 1- and 10-kb fragments of viral genomic DNA extracted from HAdV-5 suspensions treated with 15 and 25kGy were undetectable, suggesting that HAdV-5 DNA was degraded in both substrates (Fig.13). For samples irradiated at 4 kGy were visualized similar results for HAdV-5 DNA amplification between substrates, exception for 1-kb amplification fragment that was detected. Although, the proteins abundance and antigenicity were distinct between substrates. The radioresistance of wastewater substrate triggered by scavengers presence was visualized at proteins level in samples treated with 4 kGy (Fig.9). As shown in Fig. 11, the relative binding to the anti-hexon monoclonal antibody of HAdV-5 on PBS and wastewater irradiated at 4 kGy were 17% and 88%, respectively. The HAdV-5 proteins damage by gamma radiation treatments depends on the substrate, however, for HAdV-5 DNA this effect seems to be not applicable.

Extrapolating the results for viral infectivity capacity would be expected a high discrepancy between substrates. As mentioned previously, the viral infectivity log reduction for samples irradiated at 4 kGy was 6.5 for PBS and 5.0 for wastewater. The difference between substrates was about 1.5 log reduction. Considering that for 1-log represents a population reduction of 90%, the differences between substrates could be considered significant. The distinct results for PBS and wastewater follow earlier studies of HAdV-5 inactivation by gamma radiation under different environmental conditions (Pimenta et al. 2016).

Considering the obtained outputs, the new insights about the application of ionizing radiation technologies in wastewater treatment seem promising. The conventional wastewater treatment decreases the viral concentration by only 2-3 $\log_{10}$ PFU/ml (Bosh A, Pintó RM 2006). The remaining virus persists in the environment and could cause severe infections. The present study confirms that gamma radiation permits achieve higher viral reductions. According to Environmental Protection Agency from US, for drinking water disinfection treatment recommends the enteric virus removal of 99,99% (4 $\log_{10}$ PFU/ml) (US EPA 2018). The data obtained demonstrate a viral reduction > 4 $\log_{10}$ PFU/ml for both substrates using a gamma radiation dose of 4 kGy.

In this study, it was found that gamma radiation degrades viral proteins and HAdV-5 genome, resulting in viral inactivation. The understanding of viral inactivation mechanisms will provide new insights for application of gamma radiation in wastewater treatments.

Future perspectives

This specific work addressed only the inactivation of adenovirus in water substrates, being this virus the most prevalent in aquatic environments. In order to demonstrate the feasibility of ionizing radiation technology to mitigate the presence of enteric virus in environmental waters, other enteric virus should also be evaluated (e.g. Hepatitis A and E).

Additionally, a logical follow-up of this project will be to evaluate the performance of ionizing radiation in the degradation of chemical pollutants. Besides the radiolytic degradation studies, a cytotoxicity assessment should be performed to evaluate the safety of the formed radiolytic products.

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