# Degradation and inactivation of adenovirus in water by photo-electro-oxidation

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## Abstract

The present study analyzed the efficiency of the photo-electro-oxidation process as a method for degradation and inactivation of adenovirus in water. The experimental design employed a solution prepared from sterile water containing $5.10^7$ genomic copies/L (gc/L) of a standard strain of human adenovirus type 5 (HAdV-5) divided into two equal parts, one to serve as control and one treated by photo-electro-oxidation (PEO) for 3 hours and with a 5A current. Samples collected throughout the exposure process were analyzed by real-time polymerase chain reaction (qPCR) for viral genome identification and quantitation. Prior to gene extraction, a parallel DNAse treatment step was carried out to assess the integrity of viral particles. Integrated cell culture (ICC) analyses assessed the viability of infection in a cell culture. The tested process proved effective for viral degradation, with a 7 $log_{10}$ reduction in viral load after 60 minutes of treatment. The DNAse-treated samples exhibited complete reduction of viral load after a 75 minute exposure to the process, and ICC analyses showed completely non-viable viral particles at 30 minutes of treatment.

*Keywords:* adenovirus, advanced oxidation process, photo-electro-oxidation, water.

## Degradação e inativação de adenovírus na água por fotoeletrooxidação

### Resumo

O presente estudo analisou a eficiência do processo de fotoeletrooxidação como metodologia para a degradação e inativação de adenovírus em água. A concepção experimental emprega uma solução preparada a partir de água estéril contendo $5,10^7$ cópias genômicas/L (gc/L) de uma amostra padrão de adenovírus humano tipo 5 (HAdV-5), dividida em duas partes iguais, uma para servir como controle e outra tratada por fotoeletrooxidação (PEO) durante 3 horas e com uma corrente de 5A. As amostras recolhidas durante o processo de exposição foram analisadas por PCR quantitativo em tempo real (qPCR) para identificação e quantificação do genoma viral. Antes da extração de ácidos nucleicos, um passo de tratamento com DNAse paralelo foi realizado para avaliar a integridade das partículas virais. Um ensaio de qPCR integrado à cultura de células (ICC-qPCR) permitiu analisar a viabilidade de infecção em uma cultura de células. O processo mostrou-se eficaz testada para a degradação viral, com uma redução de 7 $log_{10}$ da carga viral após 60 minutos de tratamento. As amostras tratadas com DNAse exibiram redução completa da carga viral após uma exposição de 75 minutos ao processo, e a análise de ICC-qPCR mostrou partículas virais completamente não-viáveis em 30 minutos de tratamento.

*Palavras-chave:* adenovírus, processo de oxidação avançada, fotoeletrooxidação, água.

## 1. Introduction

Large volumes of treated and untreated wastewaters flow to the environment carrying contaminants that after may be transported to water bodies that laterwill serve as drinking water sources. Therefore, water disinfection practices are required to reduce the risk of human exposure to pathogenic microorganisms (Blatchley et al., 2007). Overall, the greatest microbial hazard is posed by ingestion of water contaminated with human or animal feces,

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which may carry pathogenic bacteria, viruses, protozoa, and helminth' eggs (WHO, 2011), with the potential for significant public health, economic, and societal impacts (Wong et al., 2012).

Adenoviruses are icosahedral, non-enveloped viruses containing a double-stranded deoxyribonucleic acid (DNA) genome. These viruses are present in the gastrointestinal tract; their transmission occurs via the fecal-oral route, can infect humans and other animals. The human adenoviruses (HAdVs) are responsible for conditions such as respiratory tract infections, conjunctivitis, and cystitis, among other clinical manifestations, and are excreted in extremely high numbers in the feces of infected individuals (García, 2006). HAdVs are often found especially in water bodies of medium to large urban areas (Lee and Kim, 2008) and in 2005, those viral agents were included in the U.S. Environmental Protection Agency Contaminant Candidate List 2 due their importance to public health, high resistance in the environment, ease of detection by molecular methods and frequent occurrence in a wide range of aquatic environments (USEPA, 2005a). Viruses are obligate intracellular parasites, and thus require the machinery of a host cell for replication; they cannot multiply when dispersed in the environment. Nevertheless, non-enveloped viruses are generally more resistant than prokaryotes and enveloped viruses to the chemical and physical methods employed in water and sewage treatment, such as solvents, detergents, and other compounds meant to cause their structural degradation in the environment. Hence, enteric viruses exhibit advantages over bacteria as markers of the effectiveness of water decontamination (Jiang et al., 2007). In particular HAdV have an even higher degree of resistance compared to other viruses (García, 2006).

Brazilian drinking water companies still lacks treatment systems for viral decontamination of water for human consumption, many countries have already implemented such technologies. The primary (physical) and secondary (chemical, chlorination-based) methods currently employed have low efficacy for viral decontamination of drinking water, and in view of the growing need for more efficient water treatment procedures, several techniques have been evaluated and tested as options for tertiary treatment or enhancement of secondary treatment, such as ultrafiltration, nanofiltration, and addition of an UV irradiation stage (Borchardt et al., 2003).

UV radiation-based disinfection technologies are the treatment modality most commonly reported as having the potential to inactivate the majority of waterborne virus species (Stanfield et al., 2003). However, double-stranded DNA viruses, such as the adenoviruses, are more resistant to UV disinfection than other organisms (Eischeid et al., 2009). Free chlorine application is often employed to enhance these disinfection modalities and inactivate viruses with this structure (USEPA, 2006b). However, the formation of such disinfection byproducts (DBPs) as trihalomethanes and haloacetic acids as the result of reactions between added chlorine and organic compounds present in wastewater is a major disadvantage of this process, as organochlorine

compounds have negative effects on human health (USEPA, 2005b).

An alternative group of technologies that may be used to minimize DBP formation comprises the advanced oxidation processes (AOPs) (Zhou and Smith, 2001). These processes are based on the generation of transient reactive species with high oxidation power, particularly hydroxyl radicals (·OH). Hydroxyl radicals are strongly oxidant, exhibit low selectivity, can mineralize organic compounds, and induce rapid degradation of pollutants, which enables their use against both waterborne and soil-based pollutants (Freire et al., 2000). Several processes have been tested for hydroxyl radical production, usually employing ozone (in alkaline pH), hydrogen peroxide/UV, photocatalysis, and Fenton's reagent (Freire et al., 2000). The main advantages associated with the use of AOP technologies include, for the purposes of this study, the fact that such technologies are applicable in the treatment of water containing contaminants at very low concentrations (ppb) and the fact that no residues or waste products are generated, a particularly relevant factor in that it precludes the need for supplemental treatment and disposal processes (Doménech et al., 2001).

According to the U.S. EPA (USEPA, 2006a) and Health Canada (HC, 2010), for a water treatment process or a given technology to be considered efficient, it must achieve performance levels consistent with a 99.99% (4 log) reduction in sample viral concentrations after treatment. This is the minimum reduction recommended for all water sources, including groundwater, as some publications have reported the presence of enteric viruses in underground water sources that were considered safe. At any rate, waters of all sources – particularly surface sources – require a 4-log reduction to ensure there is minimal risk of enteric viruses being present.

The present study sought to assess the efficiency of a photo-electro-oxidation process for degradation and inactivation of HAdV viral particles, on the absence of chemical disinfectants.

## 2. Material and Methods

### 2.1. Sample preparation

Four liters (4L) of a solution containing water treated by reverse osmosis (Biosystems - Biohuman new human power 1 integrate), human adenovirus type 5 (HAdV-5), cultured at in A-549 cells to a final viral load of $5 \times 10^7$ cg/mL, and 0.5% anhydrous sodium sulfate, analytical grade (Synth), for electrical conductivity. The solution was divided into two equal 2L volumes: 2L for AOP treatment (test solution) and 2L for control (control solution), which was kept under room conditions of luminosity and temperature, with no exposure to other adventitious oxidative processes.

### 2.2. Experimental photo-electro-oxidation

The experimental system (schematic diagram in Figure 1) was composed of a benchtop reactor, which was a 2L cylindrical borosilicate glass kettle containing a pair of electrodes: a dimensionally stable titanium anode
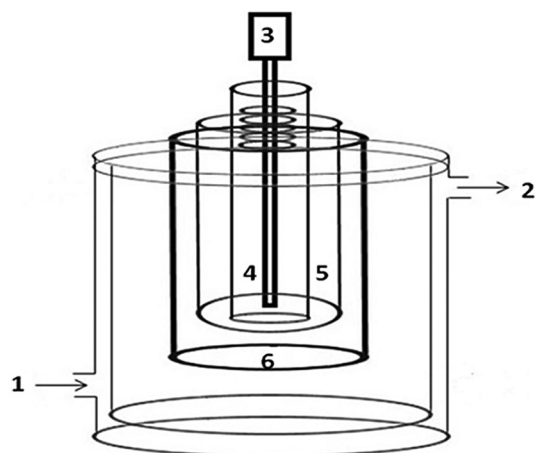
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**Figure 1.** Schematic diagram of PEO reactor, where: (1) coolant intake; (2) coolant output; (3) mercury-vapor lamp; (4) quartz bulb; (5) Ti/TiO$_2$ cathode; and (6) Ti/ $_{70}$TiO$_2$-$_{30}$RuO$_2$ anode.

(DSA) coated with Ti/ $_{70}$TiO$_2$-$_{30}$RuO$_2$ and a titanium cathode coated with titanium oxide (Ti/TiO$_2$). The electrodes were placed concentrically around the lamp, and thus remain under constant exposure to UV radiation. The source of UV radiation was a commercially available 400W mercury-vapor lamp, with the glass bulb removed and coupled to a quartz tube instead. A source of electrical current and a centrifugal pump completed the system.

The treatment duration was of 3 hours, and a 5A current was applied. Every 15 minutes during the degradation procedure, two 2-mL aliquots were collected into labeled sterile Eppendorf tubes and stored at –70 °C for later processing and molecular analysis.

### 2.3. Temperature and pH analysis

Sample pH and temperature were measured throughout the experiment and recorded every 15 minutes for assessment. Temperature was measured using a chemical thermometer (Incoterm®, Brazil) with a resolution of 1 °C, and pH was measured using universal indicator strips (Merck®, USA).

### 2.4. Molecular detection of viral genomes

Real-time polymerase chain reaction (qPCR) techniques were employed to test for and quantify human adenovirus type 5 DNA. The integrity of HAdV-5 was verified by a combination of deoxyribonuclease (DNAse) and qPCR techniques.

The extraction of viral genomes was performed using the commercial kit Invitek DNA/RNA Virus Mini Kit (Stratec®, Germany), following the manufacturer's instructions, using an initial volume of 400 mL of each concentrated sample through a silica filter. The viral DNA so obtained was stored at –80 °C for later processing.

After, the samples were subjected to DNA amplification procedures trageting a specific fragment of the HAdV genome, a conserved region of the hexon gene using the primers VTB2-HAdVCf 5'-GAGACGTACTTCAGCCTGAAT-3' and VTB2-HAdVCr 5'-GATGAACCGCAGCGTCAA-3'),

according to Wolf et al. (2010). The real-tiem polymerase chain reactions (qPCR) for detection and absolute quantification of HAdV-5 in an iQ5 Bio-Rad Real-time Thermocycler (Biorad™, USA and the results collected and analysed in iQ™5 2.1 optical system software. For the qPCR, Platinun® SYBR® Green qPCRSupermix-UDG (Invitrogen®, USA), was used: to a final volume of 25 µL per sample, 12.5 µL mix, 1 µL of each primer (20 pM), 5.5 µL sterile water and 5 µL of test DNA. Reaction cycles were composed of an initial denaturation step at 95 °C per 10 min., followed by 40X of 95 °C per 20 s, 55 °C per 1 min.. A High resolution melting (HRM) curve (from 55 to 95 °C, 0.5 °C increase per step) was made for all reactions after the amplifications to ensure the specificity of the amplicons. Standard curves were made using five serial 10-fold dilutions of titrated viral stocks, be begginig from the equivalent 6,01 x 10$^7$ genome copies/ 5 µL. All reactions and controls were made in duplicate and every assay was repeated 3 time NTC (No template control) and a negative control made of non-contaminated water were used trhoghout.

For assessment of viral integrity, in a separated sample of the same experiments, preceding the extraction step of the viral genome, the sample was treated with DNase enzyme (TURBO DNase™, Ambion®, USA) according to the methodology recommended by the manufacturer. TURBO 10X DNAse buffer solution was added concentrated 1X DNAse, containing 2 units of Turbo DNase per 1mg of DNA present, followed by incubation at 37 °C for 30 min. For inactivation of DNAse EDTA was subsequently added to a final concentration of 15mM and incubated at 75 °C for 10 min.

The virus infectivity analysis was performed by the method of Cell Culture Integrated with qPCR (ICC-RT-qPCR), using 24-well microplate, containing approximately $2.5 \times 10^5$ cells/mL in each well. 24 hours after the preparation of the plate, the samples from the reactor experiment were inoculated in cell media. After 1 h incubation at 37 °C with rotation every 15 min, the inoculum was removed and cell layers were overlaid with Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose concentration, and further incubated at 37 °C for 24 h. After incubation, the supernatant was recovered and 400 µL were used for extraction of genetic material, as described above. After, a reverse transcriptase reaction (RT) was used to generate cDNA from viral mRNA. The quantification of infectious particles HAdV qPCR was performed as described earlier.

### 2.5. Assessment of system efficiency

To analyze the efficiency of PEO for HAdV-5 inactivation and degradation, the recommendations of the U.S. EPA (USEPA, 2006b) and Health Canada (HC, 2010) agencies were followed, which recommend a 99.99% (4 log$_{10}$) reduction in viral concentration after treatment as the minimum cutoff for a treatment to be considered efficient, thus ensuring minimal risk of presence of enteric viral agents. The log$_{10}$ reduction was calculated on the basis of qPCR results.

## 3. Results and Discussion

According to Fujioka and Yoneyama (2002), as they do not require an envelope (biological membrane) to mediate their interactions with host cell surfaces and as they possess a capsid (protein shell) that resists the action of the elements, enteric viruses can resist extreme pH levels (in the 3-10 range), and may persist in the environment for several days at temperatures as high as 50 °C or for over 1 year at low temperatures. However, when testing a targeted physicochemical process meant to achieve degradation and inactivation of viral pathogens, one must control for environmental factors to obtain information on which parameters actually interfere with the process. Kim et al. (2010), in their test of Fenton's reagent for inactivation of MS2 coliphage, found that the virus was more susceptible to the AOP at lower pH levels, with approximate 0.7 log, 1.2 log, and 5.8 log inactivation of MS2 observed at pH 8.0, 7.0, and 6.0 respectively with a 10-minute reaction time.

In the present study of PEO, throughout the experiment in the reactor, the test solution remained at a pH of 6.0. The temperature of the solution increased gradually as the duration of reactor operation progressed, plateauing at 39-43 °C at 20 minutes of exposure and remaining in this range until the end of the experiment. Within this context, we believe that the temperature and pH factors, which remained stable and within range as noted above, did not influence the rate of degradation induced by the oxidation process.

The results of molecular analysis for detection and quantitation of viral load in the test and control solutions by qPCR are described in Table 1. Both the results of samples treated with DNAse, which enables identification of damaged viral particles, and those that did not undergo additional treatment before viral genome extraction.

Although the qPCR technique provides rapid, sensitive, and specific results, it cannot distinguish infectious from non-infectious viral particle; therefore, DNAse treatment was performed to enable this distinction. Girones et al. (2010) proposed that DNA viruses should undergo DNAse pretreatment with the objective of degrading all free DNA, so that the intact capsid of viral particles would protect their DNA and subsequent molecular techniques would only detect viable viral particles. The same idea was proposed by Nuanualsuwan and Cliver (2003), who suggested that samples be pretreated with protease and RNAse to distinguish viable from non-viable viral particles.

On this basis, treatment of samples with DNAse enzyme is important for a more refined detection process, with the ability to identify both genome fragments and intact virus.

The molecular analyses conducted in the present study indicate a 7 $\log_{10}$ reduction in viral concentration after 60 minutes of PEO treatment in samples not subjected to DNAse pretreatment.

Analysis of data from the samples treated with DNAse, with the objective of assessing only the intact viral particles present in the sample, revealed a need for at least 75 minutes of exposure to ensure complete removal of HAdV-5 as determined by qPCR. On the basis of the U.S. EPA (USEPA, 2006a) and Health Canada (HC, 2010) specifications, which denote a treatment as effective if it can produce a 4 $\log_{10}$ or greater reduction in viral concentration, we suggest that this process is a viable method for degradation of viral agents in water. When treatment efficiency was assessed (i.e., the percentage of particles destroyed after PEO treatment in relation to the baseline viral concentration), it was equally clear that 100% efficiency for clearance of intact particles was achieved within 60 minutes, and complete destruction of DNA remnants from non-intact particles within 75 minutes (Figure 2).
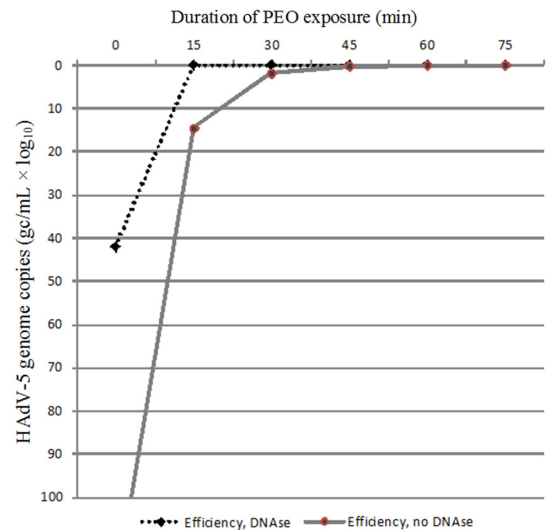


**Figure 2.** Efficiency (genome copy count after treatment vs. baseline count) of PEO treatment for degradation of HAdV-5 in water, as analyzed by qPCR, with or without DNAse pretreatment.

**Table 1.** Results of qPCR analysis of the test and control solutions with an experiment time of up to 90 minutes, considering both DNAse-treated and untreated samples.

| | | Viral load (cg/mL) x Exposure time (min) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Sample** | **0** | **15** | **30** | **45** | **60** | **75** | **90** |
| **Test Solution** | Non DNAse-treated | 5.38E+07 | 7.07E+06 | 9.16E+05 | 1.54E+05 | 0.00E+00 | 0.00E+00 | 0.00E+00 |
| | DNAse-treated | 1.45E+07 | 1.55E+04 | 1.14E+03 | 1.25E+04 | 1.20E+04 | 0.00E+00 | 0.00E+00 |
| **Control Solution*** | Non DNAse-treated | 4.45E+07 | 4.81E+07 | 5.30E+07 | 5.42E+07 | 5.17E+07 | 5.98E+07 | 8.76E+07 |
| | DNAse-treated | 3.44E+07 | 3.36E+07 | 4.80E+07 | 4.97E+07 | 4.50E+07 | 5.23E+07 | 7.44E+07 |

*Control solution was not treated with PEO, but aliquots were collected at the same time points as from the test solution.

**Table 2.** Results of ICC/qPCR analysis of test and control solutions with an experiment duration of up to 90 minutes.

| Samples | Viral load (cg/mL) vs. exposure time (min) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 15 | 30 | 45 | 60 | 75 | 90 |
| Test solution | 5.24E+06 | 1.50E+05 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 |
| Control solution* | 1.22E+07 | 7.90E+06 | 1.53E+07 | 1.05E+07 | 2.66E+07 | 1.41E+07 | 1.18E+07 |

*Control solution was not treated with PEO, but aliquots were collected at the same time points as from the test solution.

The infectious viability of HAdV in the samples was assessed by ICC/qPCR, shows that the viral particles were devoid of infectious potential from 30 minutes of treatment onward (Table 2), providing further evidence of the efficiency of the PEO process for degradation and inactivation of HAdV-5.

The quantitative results obtained in this experiment demonstrate the efficiency of this AOP in degrading and inactivating HAdV-5 under the design and conditions employed. Several investigators have assessed and proved the disinfecting capacity of semiconductor/catalyst photo-oxidation and UV light irradiation against various contaminants. For inactivation of microorganisms such as MS2 and *Bacillus subtilis* spores, Cho et al. (2011) found that the synergistic effects of a combination of several disinfection processes, such as UV irradiation/$H_2O_2$ followed by chlorination, were effective. Human enteric viruses, however, are usually more resistant than bacteria, helminths, and protozoa in the environment, and can remain viable despite exposure to chlorination and UV irradiation processes (McCormick and Maheshwari, 2004; Sauerbrei et al., 2004). However, combinations of several disinfection processes may also be applied in an attempt to degrade and inactivate viral agents. Addition of chemical disinfectants (chlorine, monochloramine, formaldehyde, etc.) to treatment processes, may lead to toxic potential of some of these reagents must be stressed. Formaldehyde, for instance, is carcinogenic in humans. Therefore, strict dose control is required. Another caveat concerns the possibility that DBPs, such as trihalomethanes, haloacetic acid, and chloroform, may form as a result of reactions between disinfectants and natural organic material present in water sources. Some DBPs are associated with the development of serious health problems, such as cancer and reproductive issues (Sirikanchana et al., 2008). In the present study, it was possible to achieve complete destruction of HAdV particles in the absence of chemical disinfectants. AOPs can be an alternative to prevent the formation of these DBPs during treatment (Zhou and Smith, 2001) while maintaining disinfecting capacity against HAdV and perhaps many other viral agents.

According to Bounty et al. (2012), who assessed HAdV inactivation by an UV/$H_2O_2$-based AOP, UV light essentially acts by creating dimers between adjacent pyrimidine bases in the genetic code, thus inhibiting viral replication and reproduction. However, the authors stress that further studies are required to elucidate the mechanisms whereby adenovirus disinfection is enhanced in the presence of hydroxyl radicals.

Several investigators have assessed the antimicrobial disinfection capacity of chemical agents. Some studies have focused on photocatalytic disinfection using $TiO_2$ powder as an additive, not only for determination of reaction parameters ($TiO_2$ concentration, light intensity, pH, etc.), but also to understand the mechanism of microorganism inactivation with this process (Cho et al., 2004).

In a study by Sauerbrei et al. (2004) comparing the sensitivity of several human adenovirus serotypes to three chemical disinfectants (peracetic acid, formaldehyde, and povidone-iodine), the authors found that, on PCR analysis, only samples treated with 0.5% peracetic acid or 0.7% formaldehyde for 60 minutes were negative for human adenovirus type 5. After 60 minutes of treatment with povidone-iodine, samples were still positive for HAdV-5 on PCR at all three tested concentrations (0.125%, 0.5%, and 2.5%). McCormick and Maheshwari (2004) assessed the disinfectant capacity of the commercially available product Virkon® S against adenoviruses type 5 and 6 and found a reduction of at least 6 $\log_{10}$ in viral concentrations after exposure to 0.09% Virkon® S (purified virus) or 0.9% Virkon® (cell concentrate) for 5 minutes. These studies, however, did not conduct comparative assessments of viable and non-viable particles, and the findings of the present work clearly demonstrates that even viral integrity and infectivity may be lost using these technologies.

## 4. Conclusions

Corroborating the findings of Bounty et al. (2012), the UV/·OH combination proved efficient in decontaminating an adenovirus-contaminated sample exposed to the PEO process. There was substantial degradation and inactivation of adenovirus in water after exposure to PEO promoted by the generation of hydroxyl radicals as stimulated by UV light irradiation and electrical current.

Although more in-depth studies using environmental samples are required, the results of the present experiment show that the PEO process is a promising option as an additional step for decontamination of enteric viral pathogens during water treatment, in view of its efficiency in adenovirus degradation and inactivation.

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