Toxicity for Aquatic Organisms of Antiretroviral Tenofovir Disoproxil

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Abstract

Tenofovir disoproxil fumarate is a prodrug, i.e. inative substance converted in vivo, after absorption to the active form de-esterified tenofovir, which acts as an inhibitor of viral reverse transcriptase. To better understand the toxic effects of these drugs in the environment, three organisms were tested, the effective concentration (EC₅₀) and inhibitory concentration (IC₅₀) of tenofovir disoproxil that resulted in 50% growth inhibition of Microcystis novacekii, 50% immobilization of Artemia salina, and 50% loss of bioluminescence of Aliivibrio fischeri were evaluated. The EC₅₀ value after 96 h of treatment for the cyanobacterium was 161.01 (156.81 - 165.21) mg·L⁻¹; the IC₅₀ value for A. salina after 24 h of treatment was 111.82 (103.18 - 120.45) mg·L⁻¹; and the IC₅₀ at 15 min for A. fischeri was 14.83 (13.87 - 15.79) mg·L⁻¹. The test organism most sensitive to the drug was A. fischeri, indicating the importance of using representative models at different trophic levels to assess the potential risk of drugs for environmental toxicity. These results highlight the possible effect of tenofovir disoproxil on decomposer organisms, which may contribute to the environmental persistence of this drug.

Keywords
Emerging Contaminant, Aliivibrio fischeri, Artemia salina, Microcystis novacekii

1. Introduction

Drug residues have been reported in several environmental systems and are considered emerging contaminants [1] [2]. In aquatic environments, these contaminants, especially more stable and persistent substances, may present a risk to
aquatic species [3]. A number of studies have indicated insufficient drug removal at water and effluent treatment stations [4] [5] [6], which highlights the importance of assessing the toxic effects of drug residues on aquatic species.

Tenofovir disoproxil fumarate (TDF) is an antiretroviral belonging to the class of nucleotide analogs used in the treatment of HIV/AIDS and viral hepatitis. TDF is a prodrug, i.e. inactive substance hydrolyzed in vivo to release the active portion of the molecule, tenofovir (TFV) (Figure 1). The prodrug is used to enhance tenofovir liposolubility and permeation across the intestinal cells.

After absorption by human organisms, TDF is de-esterified and after phosphorylation, the TFV molecule acts as an inhibitor of the viral enzyme reverse transcriptase by competing with its natural substrate, adenosine 5’-monophosphate, thus disrupting DNA synthesis and, consequently, viral replication [7]. According to Kim et al. [8], after human use of this drug, up to 80% of the TFV is eliminated in unmodified urine, indicating its potential entry into domestic and hospital sewage in its active form. TFV is a very stable molecule [9], leading to concerns about the effects of this drug on aquatic species.

Residues of more than 20 antiretrovirals drugs have been detected in various environmental compartments such as wastewater effluent, river and lakewater, hospital effluent, groundwater, drink water, landfill leachates, at varying concentrations depending on the antiviral drug [10] [11] [12]. The tenofovir has been described at low concentrations (145 - 243 ng·L⁻¹) in surface waters from South Africa [13]. A study by Al-Rajab et al. [9] in London has shown that tenofovir is persistent in soils with no evidence of transformation products or microbial based degradation. The effects of TFV on DNA synthesis and its possible interference with metabolic pathways affecting viability and cell growth of other species are unknown, and its environmental impact needs to be investigated.

Although the importance of understanding the toxicity of chemical substances for aquatic species has been recognized, there is still limited information on the effects of most drugs on the environment [14]. Several factors are associated with the toxicity of drugs in the environment, including the selectivity of the drug for the target system, its interactions with other substances in the environment and its physicochemical characteristics, as well as the intrinsic sensitivity of

![Figure 1. Metabolism of tenofovir disoproxil.](image-url)
the test system. Thus, environmental toxicity can be assessed by applying tests using different test organisms belonging to at least two trophic levels [15].

Among the most commonly used test organisms are cyanobacteria. Cyanobacteria are widely studied because of their rapid response to environmental modifications, survival in polluted aquatic environments, and dominance among other species [16]. The cyanobacterium *Microcystis novacekii* is a ubiquitous unicellular photosynthetic prokaryote [17]. According to Bicudo and Menezes (2006) [18], *Microcystis* is the cyanobacterial genus with the widest distribution in Brazil, which justifies the use of species of this genus in toxicity tests.

*Artemia salina* is a widespread microcrustacean that forms a link between planktonic communities and higher levels of food chains. *A. salina* is considered an important model for the evaluation of toxicity and possible bioaccumulation of xenobiotics in the trophic chain [19] [20]. A third model, the bioluminescence test using the bacterium *Aliivibrio fischeri*, as test organisms often used in environmental toxicity studies. *A. fischeri* is a gram-negative heterotrophic organism with widespread distribution. It is a saprophytic, free-living bacterium that uses dissolved or particulate organic matter from marine environments as a carbon source [21]. *A. fischeri* is characterized by light emission under favourable environmental conditions. *A. fischeri* is a very sensitive model for the evaluation of chemical toxicity because of its rapid response to environmental variations [15].

The objective of this study was to determine the inhibitors concentration of TDF for test organisms: *M. novacekii* (cyanobacteria), *A. salina* (microcrustaceans) and *A. fischeri* (bacteria).

### 2. Materials and Methods

#### 2.1. Chemicals Tests

The TDF sample used in this study were white dry powder, produced by Nortec Química, Brazil, (lot 507034) purity ≥ 99%, identified and certified by the Quality Control Laboratory of the Ezequiel Dias Foundation (FUNED). All solvents and reagents used were of analytical grade.

#### 2.2. Test Organisms

The species *M. novacekii* was isolated at Rio Doce State Park and then identified and maintained in culture by the Laboratory of Limnology, Ecotoxicology and Aquatic Ecology, Institute of Biological Sciences, Federal University of Minas Gerais (LIMNEA/ICB/UFMG), Brazil. *A. salina* hatching eggs (Maramar, lot 07) were purchased from a retail store in Belo Horizonte, Brazil. A freeze-dried *A. fischeri* stock was purchased from Biolux®.

#### 2.3. *M. novacekii* Growth Inhibition Tests

*M. novacekii* cultures were maintained in germination chambers at 23.0°C ± 2°C with a 12 h light/12 h dark photoperiod under light intensity (radiance of 40 - 50 μmol·m⁻²·s⁻¹) [22]. The medium used for the cultivation and in the *M. novacekii*
assays was ASM1 [23]. In total, 750 mg·L⁻¹ of 3-(N-morpholino) propanesulphonic acid (MOPS), pKₐ = 7.2 at 25°C, was added to freshly prepared and sterilized medium. The pH was adjusted to 7.0 with either 0.1 mol·L⁻¹ HCl or NaOH solution. The optical densities of the cultures were determined by visible spectrometry at 680 nm (OD₆₈₀) [24], and the correlation between the cell density (number of cells·mL⁻¹) and the absorbance was obtained by linear regression analysis.

For the M. novacekii growth inhibition tests, the OECD 201 protocol (2006) [25] was used, with some modifications. To cultures of M. novacekii with a cell density of 10⁶ cells·mL⁻¹, TDF was added to obtain nominal concentrations of 40.00, 56.00, 78.40, 109.76, 153.66, 215.12, and 300.00 mg·L⁻¹. This concentration range was defined from preliminary tests. The Erlenmeyer flasks were incubated in triplicate at 25.0°C ± 2.0°C with a 12 h photoperiod and with constant stirring. Cell growth was monitored by OD₆₈₀ of the culture, measured at the initial time (T₀) and then every 24 h. The pH was maintained in the range of 6.0 to 7.0 with MOPS buffer. Based on the growth curves, the mean growth rates were calculated, and the percentage growth inhibition curves were constructed as a function of the concentrations of TDF and TFV.

The growth rate coefficient (µ) for each culture (tests and controls) was calculated at 96 h according to the following equation:

$$\mu = \left(\frac{\ln X_1 - \ln X_0}{t_1 - t_0}\right)$$

where X₀ and X₁ are the number of cells (optical density) at 0 and 96 h, respectively; t₀ denotes 0 days; t₁ denotes the fourth day; and µ is the average specific growth rate from the period (day⁻¹).

### 2.4. A. salina Acute Immobilization Test

A method adapted from Meyer et al. (1982) [26] was used in the A. salina assay. A. salina eggs were incubated under illumination for 36 h in a 3% saline solution (pH 8.0 to 9.0) until the formation of nauplii (larvae). Ten larvae were separated and transferred to test tubes containing 5.00 mL of TDF at the following concentrations in a saline solution: 30.00, 60.00, 90.00, 120.00, 150.00, and 180.00 mg·L⁻¹. The tubes were maintained under artificial light for 24 h when non-motile larvae were counted.

### 2.5. Inhibition of Bioluminescence of A. fischeri

To evaluate the inhibition of A. fischeri bioluminescence, the tests were performed according to ABNT NBR 15411-3 (2012) [27], the instrument used in the test A. fischeri was Biofix®, ModeloLumi-10, Marcherey-nagel. The following nine serial dilutions of TDF in 2% NaCl were used: 4.38, 8.80, 13.20, 17.60, 18.48, 21.12, 26.40, 35.20, 52.80, and 70.40 mg·L⁻¹. The cultures were incubated at 15.0°C for 15 min, and a 2% NaCl solution was used as a negative control [28]. The loss of bacterial luminescence (INH%) due to the addition of toxic substances was calculated as follows:
\[
KF = \frac{IC_T}{IC_0}
\]

\[
\text{INH}\% = 100 - \left[ \frac{IT_T}{(KF \times IT_0)} \right] \times 100
\]

where \(IC_0\) and \(IT_0\) are the luminescence of the control and test samples at \(t = 0\), \(IC_T\) and \(IT_T\) are luminescence values for control and test samples measured after 15 min of exposure time, and \(KF\) is the correction factor based on the control/blank. R software was used to compare the intensities of the light emitted by the samples with the various dilutions of TDF and the control solution.

2.6. Data Analysis

The statistical dose-response regression models represent the relation between the independent variable (dose or concentration) and the dependent variable (response or effect). Log-logistic, log-normal, and Weibull models were tested using the extension package drc for the statistical software R (version 3.4.2) to estimate the best fitting function [29].

3. Results

The growth inhibition curves of \(M. \ novacekii\) exposed to TDF are shown in Figure 2(a). Based on the growth inhibition as a function of TDF concentrations (Figure 2(b)), the effective concentration (EC_{50}) of TDF for \(M. \ novacekii\) was estimated to be 161.01 (156.81; 165.21) mg·L\(^{-1}\) at 96 h of treatment. This value refers to the TDF concentration, however, the results can be expressed as (TFV), because TDF is a prodrug and easily and spontaneously de-esterified in biological medium [30] [31] for which the IC_{50} corresponded to 89.00 (86.67; 91.31) mg·L\(^{-1}\). The IC_{50} model for TFV was found considering the stoichiometric calculation of the molar mass (MM) of tenofovir disoproxil (MM = 519.4 g/mol) and the tenofovir (287.2 g/mol). The TFV is considered to be the active part of the molecule and the other concentration data is available in the literature, presented in terms of TFV.

The immobility of \(A. \ salina\) nauplii was observed after 24 h and the inhibition of luminescence of \(A. \ fischeri\) after 15 minutes of exposure to TDF. The TDF and TFV concentrations affecting the viability of these species are shown in Table 1.

4. Discussion

In this study, three trophic organisms were used: a cyanobacterium (primary producer), a crustacean (primary consumer) and a bacterium (decomposer), to estimate the environmental toxicity of tenofovir disoproxil.

4.1. Toxicity of TDF to Cyanobacterium \(M. \ novacekii\)

It was found in this study that \(M. \ novacekii\) showed resistance to TDF, tolerating concentrations higher than 100.00 mg·L\(^{-1}\). According to the criteria of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS, 2017) [32], TDF can be considered as having low acute toxicity for this strain.
**Figure 2.** Growth (a) inhibition (b) curves of *M. novacekii* exposed to increasing concentrations of TDF.

**Table 1.** Resume of point estimates (mg·L⁻¹) of IC₅₀ of TDF and TFV determined for the tests and species used in this study.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Parameters</th>
<th>IC₅₀ (mg·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. novacekii</em></td>
<td>TDF Growth inhibition (cell density) 96 h</td>
<td>161.01 (156.81; 165.21)</td>
</tr>
<tr>
<td></td>
<td>TFV</td>
<td>89.00 (86.67; 91.31)</td>
</tr>
<tr>
<td><em>A. salina</em></td>
<td>TDF Immobility 24 h</td>
<td>111.82 (103.18; 120.45)</td>
</tr>
<tr>
<td></td>
<td>TFV</td>
<td>61.83 (57.05; 66.59)</td>
</tr>
<tr>
<td><em>A. fischeri</em></td>
<td>TDF Luminescence inhibition 15 min</td>
<td>14.83 (13.87; 15.79)</td>
</tr>
</tbody>
</table>
|               | TFV                                         | 8.20 (7.67; 8.73)
Cyanobacteria as well as bacteria possess some metabolic systems similar to those of eukaryotic cells, with most enzymatic pathways present, including esterase enzymes [33]. Therefore, the toxicity of the antiviral drug may be expressed as that of TFV, because TDF is a prodrug. The hydrolysis product (TFV) is the bioactive form of the drug binds reverse transcriptase by disrupting DNA synthesis drug [7]. Wood et al. (2015) [13] detected in the environment the active drug TFV. In this case, there is a considerable reduction in the growth inhibitory concentrations, causing the TFV to be classified as category 3, i.e., having moderate environmental toxicity. The expression of toxicity in terms of TFV is also important because TDF is de-esterified in the human body and excreted as TFV (c.a. 80% of TDF absorbed) that can contaminate domestic sewage.

The results obtained in this study are similar to those reported by Russo et al. (2018) [34] for antiretroviral nucleoside analogues stavudine and zidovudine belonging the same group of TDF. According to those studies, these drugs presented a weak inhibitory activity on Raphidocelis subcapitata growth, microalga specie very used in ecotoxicological studies.

The resistance of cyanobacteria to antivirals can be explained by morphological aspects such as mucilaginous production and the presence of cell walls. The phytoplankton biomass can also act as a biosorbent for hydrophobic organic pollutants [35] [36] [37]. The extracellular polymeric substances (EPS) present in mucilaginous layer, containing functional groups such as amine, carboxyl, and phosphate [38] can provide binding sites for the biosorption of hydrophilic substances. Thus, EPS can retain xenobiotics in the mucilaginous layer, protecting the cell from toxic compounds.

4.2. Toxicity of TDF to A. salina

In this study, the IC50 of TDF for A. salina was lower than the estimated EC50 for M. novacekii. Other studies have already reported the same results in terms of the higher sensitivity of the A. salina model compared to a primary producer as a test organism [39] [40].

Although single-celled species are apparently more sensitive to xenobiotics, certain aspects of A. salina may explain its high sensitivity. A. salina is a known filtering organism that is able to bioaccumulate xenobiotics [38] [41]. This process can lead to higher intra-organism concentrations of xenobiotics than external concentrations. Furthermore, TDF may be more bioavailable in saline than in the ASM1 medium and may cross membranes, thereby increasing the concentration of the intracellular drug. We supposed that in intracellular medium, esterases of A. salina can hydrolyse TDF, releasing TFV.

Although the mechanism of toxicity of TFV to the crustacean A. salina has not been elucidated, many enzymes involved in the metabolic process of this species exhibit increased activity during larval development, including the adenosine triphosphatase activated by sodium and potassium (Na, K-ATPase) [42] [43] and hydrolases [44]. Ahmed-Ouameur et al. (2005) [45] have shown
that AZT, another viral reverse transcriptase inhibitor, binds to Na, K-ATPase \textit{in vitro}. This enzyme reaches very high levels in nauplii, and it is the main physiological mechanism of osmoregulation. Any disturbance in the activity of this enzyme can lead to organism death. It is possible that TFV may also interact with this crustacean enzyme, causing toxic effects. The elucidation of TFV toxicity mechanisms for this species was not the aim of this study, however, it is important to consider the characteristics of model organisms that may explain the sensitivity of the test.

4.3. Toxicity of TDF to \textit{A. fischeri}

\textit{A. fischeri} was very sensitive to TDF, which is an important aspect to consider. TFV has great chemical stability in the environment and is biodegraded slowly \cite{46} \cite{47} \cite{48}. \textit{A. fischeri} is a saprophyte bacterium, therefore, it can be proposed that TFV can potentially affect biological processes in aerobic treatment systems.

An initial increase in bioluminescence was observed in the test with \textit{A. fischeri}. This effect has been described for several species under stress conditions and is attributed to the hormesis phenomenon, a process that stimulates the metabolism of toxic chemicals at very low concentrations. Mennillo \textit{et al.} (2018) \cite{49} investigated the ecotoxicological properties of ketoprofen, an anti-inflammatory drug and reported a hormetic effect on \textit{A. fischeri} exposed to ketoprofen.

Although \textit{A. fischeri} is more sensitive to TDF than the other organisms in this study, other researchers have obtained different results. Maselli \textit{et al.} (2015) \cite{50} compared the sensitivities of different ecotoxicity tests for crude and treated effluents from the pharmaceutical industry and found that the crustacean \textit{Daphnia similis} and the microalga \textit{Raphidocelis subcapitata} were more sensitive as indicators of toxicity than \textit{A. fischeri}. The same observation was made by Minagh \textit{et al.} (2009) \cite{51} regarding the toxicity of sertraline to \textit{A. fischeri} and \textit{Daphnia magna}, with the latter being the more sensitive species.

The variation in the toxicity of a chemical compound for different test organisms species is due to differences in the interaction between the substance and the target system of each species studied, the time of exposure and the ability of the organisms to recover during and after exposure. Therefore, to evaluate environmental toxicity, the use of several test organisms is recommended because species maintain different mechanisms of resistance to xenobiotics and the use of organisms from different trophic levels may reveal a cascading effect of a chemical on the aquatic ecosystem.

Environmental studies on the impact of TFV on aquatic biota have not been described in the literature, despite the presence of the drug at low concentrations (145 - 243 ng·L$^{-1}$) in surface waters in South Africa \cite{13}. It is emphasized that no risk is expected for this drug at the concentrations already detected in the environment. However, investigations about toxic effects of this drug are very im-
5. Conclusions

According to the organism bioindicators used to estimate TDF toxicity, *A. fischeri* was the most sensitive model to the toxic effects of TDF, while *M. novacekii* (cyanobacteria) and *A. salina* (crustacean) were moderately sensitive to the drug.

This result is worrying because *A. fischeri* is saprophytic; thus, this can indicate possible injurious effects on the other decomposer organisms, possibly inhibiting drug biodegradation. TFV is a very stable molecule, and biodegradation inhibition can contribute to enhancing the persistence of this drug in the environment.

Although TDF has shown moderate toxicity to the other organisms tested, algae and crustaceans, this does not mean that the drug is environmentally safe. It is necessary to consider the stability and low solubility of TFV in aqueous medium. This drug could accumulate in the environment and bioaccumulate in aquatic organisms. In addition, it should be emphasized that active pharmaceutical compounds are normally present as mixtures in aquatic environments. In HIV treatment, TDF is mainly administered in association with other antiviral drugs. Thus, additive or potentiating toxic effects can still occur, increasing damage to the ecosystem.

The present results highlight that a battery of bioassays representing different trophic levels is fundamental in predicting the toxicity of drugs, regardless of whether significant variations can occur between species tests. Furthermore, the tests provide the information required to define safe levels of drugs for living organisms in the environment. In addition, the physicochemical properties of TFV and the toxicity to the species tested in this study indicate the need to investigate the presence of this drug on the environment, its persistence after effluent treatment and potential disturbances in the equilibrium of aquatic ecosystems.

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Ethical Approval

This article does not contain any studies with human participants or vertebrate
animals performed by any of the authors.

**Conflicts of Interest**

The authors declare that they have no conflict of interest.

**References**


Chemicals.


