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Revealing the toxicity of lopinavir- and ritonavir-containing water and wastewater treated by photo-induced processes to *Danio rerio* and *Allivibrio fischeri*

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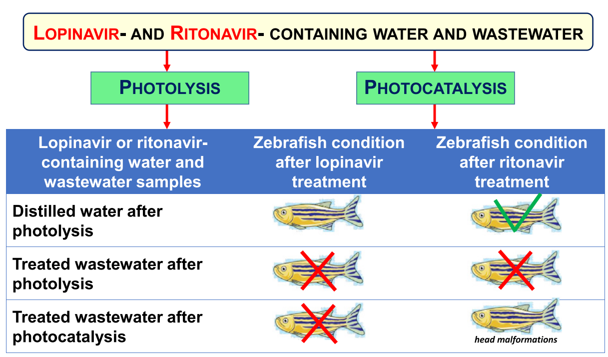
HIGHLIGHTS

- Lopinavir and ritonavir after photo-induced treatment affected zebrafish and bacteria.
- Photo-induced treatment of lopinavir-containing wastewater showed a lethal effect.
- Lopinavir should be removed from water before any photo-induced treatment.
- Photo-induced processes of ritonavir removal from water reduced the toxicity.
- Lopinavir and ritonavir are priority antiviral drugs that need to be monitored.

GRAPHICAL ABSTRACT



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ABSTRACT

In coronavirus disease 2019 (COVID-19), among many protocols, lopinavir and ritonavir in individual or combined forms with other drugs have been used, causing an increase in the concentration of antiviral drugs in the wastewater and hospital effluents. In conventional wastewater treatment plants, the removal efficiency of various antiviral drugs is estimated to be low (<20%). The high values of predicted no-effect concentration (PNEC) for lopinavir and ritonavir (in ng·L^{-1}) reveal their high chronic toxicity to aquatic organisms. This indicates that lopinavir and ritonavir are current priority antiviral drugs that need to be thoroughly monitored and effectively removed from any water and wastewater samples. In this study, we attempt to explore the impacts of two photo-induced processes (photolysis and photocatalysis) on the toxicity of treated water and wastewater samples containing lopinavir and ritonavir to zebrafish (*Danio rerio*) and marine bacteria (*Allivibrio fischeri*). The obtained results reveal that traces of lopinavir in water under photo-induced processes may cause severe problems for *Danio rerio*, including pericardial edema and shortening of the tail, affecting its behavior, and for *Allivibrio fischeri* as a result of the oxygen-depleted environment, inflammation, and oxidative stress. Hence, lopinavir must be removed from water and wastewater before being in contact with light. In contrast, the photo-induced processes of ritonavir-containing water and wastewater reduce the toxicity significantly. This shows that even if the physicochemical parameters of water and wastewater are within the standard

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requirements/limits, the presence of traces of antiviral drugs and their intermediates can affect the survival and behavior of *Danio rerio* and *Allivibrio fischeri*. Therefore, the photo-induced processes and additional treatment of water and wastewater containing ritonavir can minimize its toxic effect.

1. Introduction

In the last two years, the inclusion of antiviral drugs in the prophylaxis of coronavirus disease 2019 (COVID-19) has been widespread (Riva et al., 2020). COVID-19 that triggered the pandemic since March 2020 (Ellinger et al., 2021; Marcatili et al., 2021; Umar et al., 2021) is an infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to the beta-coronavirus family (Kirmani et al., 2021) and causes acute respiratory infections associated with high mortality.

Since then, significant global efforts have been made to stop the COVID-19 pandemic. The use of antiviral drugs has been one of the promising strategies to reduce virus transmission. As the estimated average time for the approval of new antiviral agent is more than 10 years, repurposing the existing antiviral drugs has been employed to the development of treatment for SARS-CoV-2. A variety of known drugs, such as nitazoxanide, remdesivir, camostat, nafamostat, mefloquine, papaverine, favipiravir, darunavir, hydroxychloroquine, chloroquine, ivermectin, lopinavir, and ritonavir were tested individually and in combination with other drugs to consider their proven activity against other viruses like SARS-CoV-2, SARS-CoV, MERS-CoV, Ebola virus, HIV, and *Plasmodium* spp. (Ellinger et al., 2021). However, their treatment efficiency was quite diverse (Choudhary et al., 2021; Shamsi et al., 2021). Although the efficacy of some antiviral drugs is still under question (e.g., unknown optimal dosage and treatment duration), they were approved for the emergency treatment of COVID-19 (Rodgers et al., 2021).

Lopinavir (LPV) and ritonavir (RNV) (Ellinger et al., 2021) are among the antiviral drugs intensively tested in the therapy of human immunodeficiency virus (HIV) infections. LPV is an antiretroviral protease inhibitor ($C_{37}H_{48}N_4O_5$, molecular weight - 628.80 g·mol^{-1}, $\log K_{OW} = 5.94$), which is usually used in the HIV treatment in combination with RNV - HIV protease inhibitor ($C_{37}H_{48}N_6O_5S_2$, molecular weight - 720.94 g·mol^{-1}, $\log K_{OW} = 6.29$) to increase its half-life by inhibiting its degradation by cytochrome P450 3A4 (Krumm et al., 2021). LPV and RNV are used in the treatment of HIV and to prevent postnatal mother-to-child HIV transmission through breastfeeding (Nagot et al., 2021). Due to the effect of LPV and RNV on the proteolysis in the coronavirus replication cycle, the combination of lopinavir and ritonavir was recommended for the treatment of pneumonia caused by SARS-CoV-2 in China (Choudhary et al., 2021). Considering the data presented by Carpagnano et al. (2021), the increased usage of LPV-RNV during the COVID-19 pandemic resulted in the increase of their concentrations in the wastewater and hospital effluents. Due to the available data on the presence of antiviral drugs in the wastewater and hospital effluents, a few attempts have been made to estimate the predicted environmental concentrations (PEC) of antiviral drugs because of the increased usage in the treatment of COVID-19 (Kumari and Kumar, 2021). Because of the ionic structure of antiviral drugs, a high bioaccumulation factor of RNV (1.32 L·Kg^{-1}) and LPV in fishes was estimated, implying that hydrophobic or lipophilic chemicals are favorably bioaccumulated in fish tissues (Kumari and Kumar, 2021). It was estimated that up to $1.89 \cdot 10^{-4}$ μg·L^{-1} and 239 μg·kg^{-1} of LPV and $2.08 \cdot 10^{-5}$ μg·L^{-1} and 169 μg·kg^{-1} of RNV can be observed in river water and fishes, respectively, if those antiviral drugs are used in the treatment of COVID-19 at the present level (Kumari and Kumar, 2021). The modeling study based on quantitative structure-activity relationship suggests that the removal efficiency of various antiviral drugs in conventional wastewater treatment plants (WWTPs) is relatively low (<20%), resulting in the presence of drug residues and their metabolites in the secondary effluents from the WWTPs (Kuroda et al., 2021). However, some predictions are contrary (Kuroda et al., 2021). LPV and RNV are excreted mostly with feces in 22% and 37% as a pristine compound, respectively, whereas the removal efficiency in the WWTPs is modeled to be about 92% (Kuroda et al., 2021). However, a study conducted by Abafe et al. (2018) revealed significantly lower removal rates for LPV (43%) and RNV (71%), while in the WWTPs, 3200 ng·L^{-1} RNV and 3800 ng·L^{-1} LPV were detected.

The predicted no-effect concentration (PNEC) values for LPV (4.7 ng·L^{-1}) and RNV (2.9 ng·L^{-1}) (Kuroda et al., 2021) indicate the risk of their chronic toxicity to aquatic organisms due to exceptionally high hydrophobicity of RNV (Kuroda et al., 2021). The PNEC value for a dose used in the COVID-19 infection for the fish consumption exposure is estimated to be 83.5 μg·L^{-1} for LPV and 40.7 μg·L^{-1} for RNV (Kuroda et al., 2021). If the improperly treated effluents containing LPV and/or RNV are discharged into the surrounding water bodies, they can lead to the transfer of antiviral drugs in the food chain, posing a serious risk to human health (Kumari and Kumar, 2021). Additionally, the estimated high values of risk to aquatic organisms (313.90 for RNV and 22.53 for LPV) indicate a pressing need to rigorously monitor and efficiently remove these antiviral drugs from water and wastewater (Kumari and Kumar, 2021). All the presented data points to the additional treatment of wastewater containing antiviral drugs are needed to develop new effective methods for the removal of antiviral drugs from wastewater.

The present study aims at exploring the effectiveness of two photo-induced processes (photolysis (Hojamberdiev et al., 2020) and photocatalysis (Czech and Hojamberdiev, 2016; Czech et al., 2020)) in the decontamination and detoxification of water and wastewater contaminated with LPV and RNV. Two standardized bioassays with fish (*Danio rerio*) and marine bacteria (*Allivibrio fischeri*) were used for the toxicity assessment. Recently, zebrafish (*D. rerio*) has gained a significant attention as a model organism for studying the in vivo effects of various treatments on vertebrates due to its sensitivity, simplicity (compact genome), affordability, and fully developed biological structures in a short time (US EPA, 2019). Microtox® is a system using sensitive bioluminescent bacteria (*A. fischeri*) for in vitro studies of toxic substances in different matrices.

2. Experimental

2.1. Photolytic and photocatalytic tests

The tests were performed initially in distilled water and then in treated wastewater from municipal wastewater treatment plant (WWTP) in Lublin, Poland (51°15′N 22°34′E). The WWTP is a mechanical and biological wastewater treatment plant with the increased removal of biogenic compounds (nitrogen and phosphorus) from wastewater. The total amount of wastewater flowing into the WWTP in 2020 was 71,907 m^3 per day (average). The treated wastewater (tWW) was characterized by a low level of COD (chemical oxygen demand) - 27.3 mg·L^{-1}, BOD (biological oxygen demand) - 4.9 mg·L^{-1}, total solids - 6.0 mg·L^{-1}, total phosphorous - 0.28 mg·L^{-1}, and total nitrogen - 8.404 mg·L^{-1}.

The toxicity of water and wastewater samples, which contained lopinavir (CAS no.: 192725-17-0, ≥98%, Merck) and ritonavir (CAS no.: 155213-67-5, ≥98%, Merck), treated by photolysis and photocatalysis was studied using *Danio rerio* and *Allivibrio fischeri*. The photolysis of water and wastewater samples containing antiviral drugs was carried out using a Heraeus photoreactor under visible light irradiation (medium-pressure mercury lamp (150 W) with the intensity of 7.31–7.53 mW·cm^{-2} and a photon flux of $20.83 \cdot 10^{19}$ m^2·s^{-1}) for 90 min. The initial concentrations of lopinavir and ritonavir were 10 mg·L^{-1} in distilled water and labeled as L_i and R_i, respectively, and the final solutions after 90 min of visible light irradiation were labeled as L_{90} and R_{90}, respectively. The initial concentrations of lopinavir and ritonavir were also 10 mg·L^{-1} in treated wastewater (tWW) and labeled as L_{tWWi} and R_{tWWi}, respectively. The final solutions after 90 min of visible light irradiation were labeled as L_{tWW90} and R_{tWW90}, respectively.

To verify the effect of advanced oxidation treatment of tWW, the photocatalytic test was also conducted using a photochemical reactor (0.7 L) equipped with a lamp in the center of the reactor. The light source used was the above-mentioned mercury lamp (150 W) with the intensity centered at 500–550 nm, the intensity of 7.31–7.53 $mW \cdot cm^{-2}$, and the photon flux of $20.83 \cdot 10^{19}$ $m^2 \cdot s^{-1}$. In the photocatalytic test, 400 $mg \cdot L^{-1}$ WO_3 (99.9% Merck) was used as the photocatalyst, and the solutions were labeled as L_{WO3} and R_{WO3}. First, the WO_3 powders were dispersed in lopinavir- or ritonavir-containing tWW (10 $mg \cdot L^{-1}$) under magnetic stirring and maintained for 30 min in the dark to reach adsorption-desorption equilibrium. Afterward, the light was turned on, and the photocatalytic reaction was carried out for 90 min.

The concentration of antiviral drugs was analyzed using an Agilent LC-DAD chromatograph (1260 Infinity II) equipped with an InfinityLab Poroshell 120 EC-C18 column (3.0 × 150 mm, 2.7 μm), using 80:20 *v*/v MeOH:H_2O as a mobile phase, T = 30 °C, and determination at $\lambda_{Ritonavir}$ = 238.4 nm, (R_T = 2.98 min, $\lambda_{Lopinavir}$ = 210 nm, R_T = 3.9 min). The water and wastewater samples treated by photolysis and photocatalysis were characterized by analyzing their physicochemical parameters: electric conductivity (EC), pH, dissolved oxygen (DO), and ammonium nitrogen content (N-NH_4) using an HQ430d Benchtop Single Input, Multi-Parameter Meter (Hach).

2.2. Toxicity tests

To determine the acute toxicity of water and wastewater samples, Fish Embryo Acute Toxicity (FET) test was conducted according to the OECD Guidelines for testing chemical substances (test no. 236) on zebrafish (*Danio rerio*). The test determines acute or lethal toxicity of chemicals on embryonic stages of zebrafish. Briefly, the fertilized zebrafish eggs were exposed to the photocatalytically treated water samples for 96 h. The photocatalytically treated water samples were diluted to the concentrations of 25, 50, 75, and 100% with an E3 medium. The E3 medium contained 5 $mmol \cdot L^{-1}$ NaCl, 0.17 $mmol \cdot L^{-1}$ KCl, 0.33 $mmol \cdot L^{-1}$ $CaCl_2$, and 0.33 $mmol \cdot L^{-1}$ $MgSO_4$ without methylene blue and had a pH value of 7.2. The system applied in this ecotoxicity test was static since the change in the sample concentration did not exceed 20% of the initial concentration value.

The toxicity test was conducted in 24 well plates, 5 embryos per well, 10 per group, in triplicate. The well plate was covered and kept in an incubator at 28 ± 0.5 °C under the light/dark conditions (12 h/12 h). The growth of the fertilized eggs was monitored every 24 h for four visual observations: (i) the coagulation of the fertilized eggs, (ii) the lack of the somite formation, (iii) the lack of the detachment of the tail-bud from the yolk sac, and (iv) the lack of heartbeat. The presence of any of these observations was recorded as an indicator of lethality. At the end of the exposure period (at 96 hpf - hour post-fertilization), acute toxicity was determined based on the positive outcome in any of the four visual observations recorded. Furthermore, the digital photographs of zebrafish from each group were taken at the final period to monitor the occurrence of any developmental malformations. A SteREO Discovery.V8 microscope (ZEISS) was used for visual observations.

Toxicity towards marine bacteria (*Allivibrio fischeri*) was evaluated based on the inhibition of bioluminescence using the Microtox® test. The luminescence inhibition was determined after 5 min and 15 min of exposure of *A. fischeri* to the aqueous samples according to the standard protocol (Microtox ®, 1995) in a Microtox M500 analyzer with the Omni software. The tests using photolytically and photocatalytically treated wastewaters were performed, and the results indicated no changes in *Danio rerio*. Similarly, wastewaters after both treatments without tested drugs revealed no toxicity to *Allivibrio fischeri*. The obtained data are expressed with the standard deviation (S.D.). The obtained data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test to assess the significance of differences between the control and treated groups.

3. Results and discussion

3.1. Physicochemical parameters of tested solutions

The fate of all antiviral drugs involves the metabolism in the body and then excretion. LPV and RNV are excreted mainly with feces: 22% LPV as an unchanged drug and 37% RNV as an unmetabolized fraction (Kuroda et al., 2021). The effect of photolytic and photocatalytic treatment on the presence of LPV and RNV and their decomposition products and physicochemical parameters of water and wastewater samples was studied.

The photolysis of LPV and RNV slightly reduced the electrical conductivity of aqueous solutions (Table 1, Fig. 1A), whereas the photocatalytic treatment led to a significant reduction in electrical conductivity and the related amount of suspended solids. The high values of electrical conductivity indicate water pollution (Teixidó et al., 2019). It implies that the amount of diluted organic carbon was reduced (Poulios et al., 2000), confirming the removal of organic pollutants. By comparing the electrical conductivity of water containing the traces of LPV and RNV by-products, higher electrical conductivity was noted for RNV due to the presence of sulfates (the decomposition product of S from RNV molecule). The revealed differences in the electrical conductivity of tWW and RNV- and LNV-spiked tWW was not statistically important. However, the photo-induced processes of RNV-containing tWW reduced electrical conductivity significantly, indicating that a lower concentration of accessible ions was present in the solution. This is the confirmation that photocatalysis is an effective process for the total mineralization of organic compounds into CO_2 and H_2O. The higher electrical conductivity of photocatalytically treated tWW containing RNV than LNV-containing tWW is caused by the decomposition of sulfur into sulfates.

Other physicochemical parameters of aqueous solutions confirmed the decomposition of LPV and RNV and their intermediates to some extent.

Table 1
Physicochemical parameters of tested solutions.

	Concentration of antiviral drugs	EC[a]	N-NH_4	DO	pH
	[$mg \cdot L^{-1}$]	[$\mu S \cdot cm^{-1}$]	[$mg \cdot L^{-1}$]	[$mg \cdot L^{-1}$]	[−]
L_i	10 ± 0.1	9.3 ± 0.3	0	10.69 ± 0.39	5.27 ± 0.19
L_{90}	0.20 ± 0.01	6.2 ± 0.2	0	9.55 ± 0.35	5.78 ± 0.21
tWW	0	1310 ± 48.3	129 ± 4.8	10.91 ± 0.4	8.36 ± 0.31
L_{tWWi}	10 ± 0.15	1335 ± 49.2	70 ± 2.6	8.19 ± 0.30	8.16 ± 0.30
L_{tWW90}	1.14 ± 0.01	1286 ± 47.4	135 ± 5.0	9.75 ± 0.36	7.78 ± 0.29
L_{WO3}	0.23 ± 0.02	640 ± 23.6	32 ± 1.2	8.85 ± 0.33	7.74 ± 0.29
R_i	10 ± 0.1	9.5 ± 0.3	0	9.77 ± 0.36	6.41 ± 0.24
R_{90}	0	11.57 ± 0.4	0	9.73 ± 0.36	5.36 ± 0.20
tWW	0	1310 ± 48.3	129 ± 4.8	10.91 ± 0.4	8.36 ± 0.31
R_{tWWi}	10 ± 0.15	1261 ± 46.5	79.2 ± 2.9	8.96 ± 0.33	8.17 ± 0.3
R_{tWW90}	0.82 ± 0.01	791 ± 29.2	130 ± 4.8	9.43 ± 0.34	7.93 ± 0.29
R_{WO3}	0	772 ± 28.5	38.7 ± 1.4	9.14 ± 0.34	7.55 ± 0.28

[a] EC- electrical conductivity [$\mu S \cdot cm^{-1}$], N-NH_4 - ammonium nitrogen [$mg \cdot L^{-1}$]. DO – dissolved oxygen [$mg \cdot L^{-1}$].

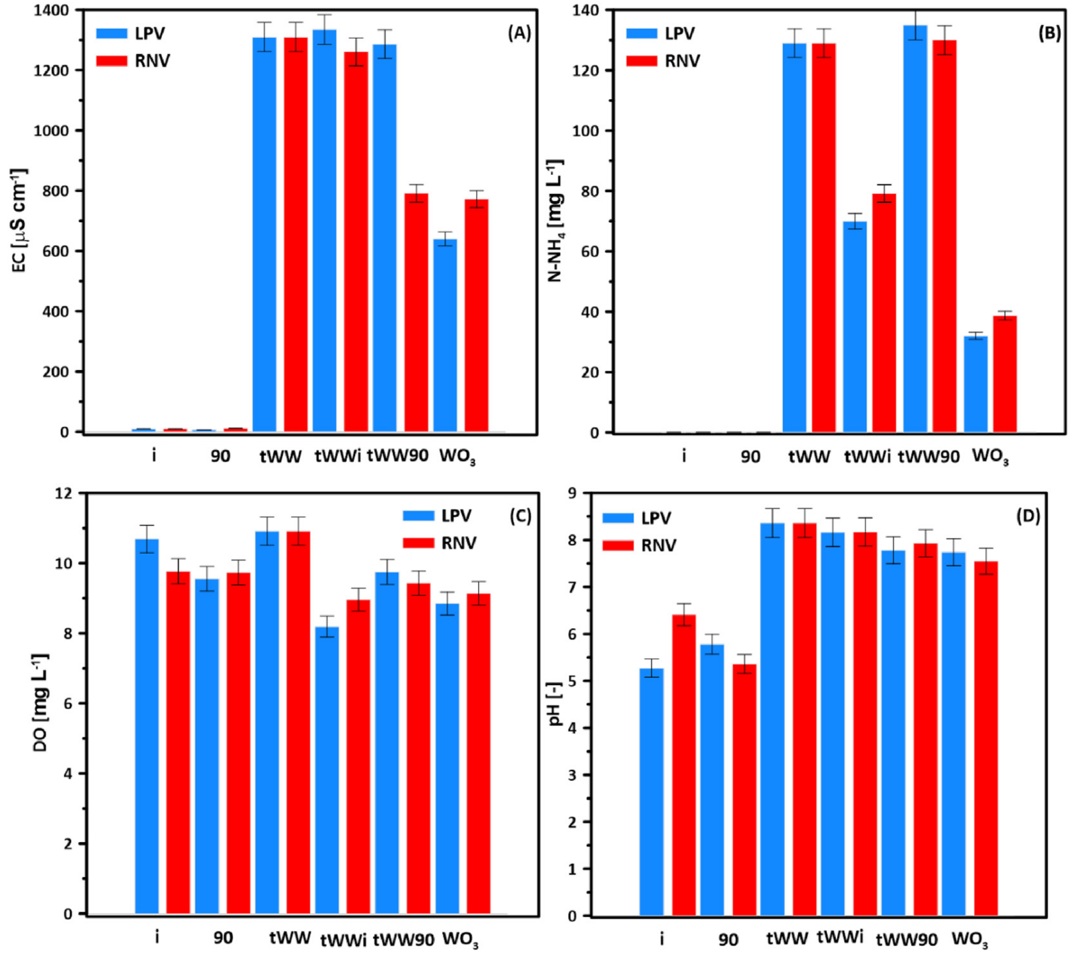

Fig. 1. Physicochemical parameters of water and wastewater samples: (a) electric conductivity, (b) amount of ammonium-nitrogen, (c) amount of dissolved oxygen, and (d) pH.

The photocatalysis reduced the amount of nitrogen in the form of NH_4^+ ion up to 54% of the initial value (t_{WWi}) (Fig. 1B). The results may arise from the fact that ammonia exists in equilibrium as both molecular ammonia (NH_3) and ammonium ion (NH_4^+). Their relative concentration depends on the pH and temperature. In the solutions with pH > 8, the amount of molecular ammonia was higher but not included in NH_4^+-N sum. The amount of determined DO was lower after all applied processes of the treatment of water and wastewater from LPV (Fig. 1C). The photolysis of water containing LPV or RNV did not lead to significant changes in the pH of slightly acidic solutions. However, the treatment of tWW with LPV or RNV resulted in a slightly increased pH, and the pH of the final solutions was neutral (Fig. 1D).

3.2. Toxicity of treated water samples to Danio rerio

Considering their negative impact on algae, daphnia, and fish, antiviral drugs were reported to be the most hazardous and toxic pharmaceuticals (Sanderson et al., 2004; Zhou et al., 2015). Narcosis is a main toxic effect of LPV for aquatic organisms, whereas the toxicity of RNV and its metabolite stems from its acetylcholinesterase, inhibiting the effect, according to the prediction by VEGA (a tool used for the prediction of toxicity) (Kuroda et al., 2021). The Ecological Structure Activity Relationships (ECOSAR) Predictive Model predicted the ecotoxicity (LC_{50} for fish after 96 h exposure) of LPV and RNV at the level of 0.099–0.744 $mg{\cdot}L^{-1}$ and 0.061–0.601 $mg{\cdot}L^{-1}$, respectively (US EPA, 2019).

The physicochemical parameters of LPV and RNV (e.g., $\log K_{ow} \sim 6$) indicate high bioaccumulation potential, and the low removal rates in the WWTPs were responsible for the estimation of a significant hazard to fish. The hazard quotient of antiviral drugs is expected to reach 10,000 (Sanderson et al., 2004). The mortality of *D. rerio* was one of the endpoints evaluated in our study (Fig. 2). As shown in Fig. 2, the fish embryos survived in the concentrated solutions (labeled as 100%), only in spiked treated wastewater with LPV (L_{tWWi}), and in irradiated water with LPV (L_{90}). Interestingly, when LPV-spiked tWW was irradiated or photocatalytically treated, the products of LPV photodecomposition were lethal to *D. rerio.* Fish exposed to 75% of aqueous solutions (L_{tWW90} and L_{WO3}) showed some malformations, and mainly scoliosis was noted. Considering the effect of tested aqueous solutions on the physical conditions of *D. rerio*, it can be seen that any malformations were noted at 50%-diluted aqueous solutions. The presence of 5 $mg{\cdot}L^{-1}$ LPV in distilled water caused a mandibular malformation and pericardial edema. *D. rerio* in 75%-diluted aqueous solution of tWW revealed an eye malformation. Although the survival of *D. rerio* in irradiated LPV-containing solution was increased, the tail autophagy was observed. The length of the tail was significantly shorter, at the 50%- and 75%-diluted solutions, the length of the tail was reduced by 50–60%. This may imply that traces of LPV in water subjected to visible light irradiation may cause severe problems for fishes, including pericardial edema and shortening of tail, which affects their behavior, as a result of the oxygen depletion environment (the presence of organics) (Hashiguchi et al., 2021), inflammation (Mohan Prakash et al., 2020) and oxidative stress (Snega Priya et al., 2021). Another interesting observation was noted when the non-toxic LPV-spiked tWW after 90 min of visible light irradiation (sample labeled as L_{tWW90}) exhibited some toxicity, and scoliosis in fish was detected. In the 50%-diluted aqueous solution, the embryos were shorter (23%) and scoliosis was observed (Fig. 3A). A similar effect was observed in photocatalytically treated solution (L_{WO3}). The data imply that the products generated after irradiation of LPV-containing tWW

Fig. 2. Effects of (A) LPV and (B) RNV in the water and treated wastewater samples. tWW is treated wastewater; L_i and R_i are LPV and RNV (10 mg·L^{-1}) in distilled water, respectively; L_{90} and R_{90} are LPV and RNV in distilled water after visible light irradiation for 90 min, respectively; L_{tWWi} and R_{tWWi} are LPV and RNV (10 mg·L^{-1}) in treated wastewater, respectively; L_{tWW90} and R_{tWW90} are LPV and RNV in treated wastewater after visible light irradiation for 90 min, respectively; L_{WO3} and R_{WO3} are LPV and RNV in photocatalytically treated wastewater, respectively; *EM*, *HM*, and *MM* are eye, head, and mandibular malformations, respectively; *PE* is pericardial edema; *S* is scoliosis, and *TA* is tail autophagy.

are toxic and cause sublethal changes to tested organisms (Rothe et al., 2021), and LPV should be removed from water and wastewater before light exposure.

The survival rate of *Danio rerio* in RNV-containing water samples was slightly different from that observed for LPV-containing water samples (Fig. 2b). The most toxic (e.g., lethal) was RNV-containing tWW before any additional treatments (R_{tWWi}). The surprising results indicate that even the PNEC for ritonavir was indicated to be toxic to fish, such visible effect was not observed. Nevertheless, it does not exclude any non-lethal changes in the tested organism, including hematological alteration, inflammatory response, altered gut bacterial community, and inhibition of PI3K signaling pathway (Yang et al., 2020). The fish embryos exposed to the highest concentration of RNV-containing solution revealed the highest lethal or sublethal changes. As the number of both dead embryos and noted malformations was strictly linked to the increased concentration of aqueous solution, it can be assumed that the severity of malformations had possibly led to deaths at 100% concentration of aqueous solution (Tenorio-Chávez et al., 2020). RNV-containing distilled water (R_i and R_{90}) did not affect the tested organism. The photolysis of RNV-containing tWW (R_{tWW90}) reduced toxicity significantly. The products of RNV oxidation include protonated amine formed from the CN bond cleavage and alcohol from hydrolysis of the carbamate bond (Rao et al., 2010). The sublethal changes (e.g., scoliosis - modified rope structure) were observed when the fish was exposed to 75%-diluted effluent. The photocatalytic treatment (R_{WO3}) was the most efficient process in toxicity reduction, and the head malformation was observed only at 100% concentration of effluent. Similarly, the multiphase photocatalysts exhibited a 95% efficiency in the photocatalytic removal of ritonavir within 15 of visible light irradiation, whereas 60 min of visible light irradiation was necessary to achieve 95% efficiency in the photocatalytic removal of lopinavir (Hojamberdiev et al., 2022). Several malformations in the fish and even teratogenic effects were observed for hospital effluents (Tenorio-Chávez et al., 2020) due to the presence of metals and drugs. The obtained data indicate that even if the physicochemical parameters of water and wastewater are within the standard requirements/

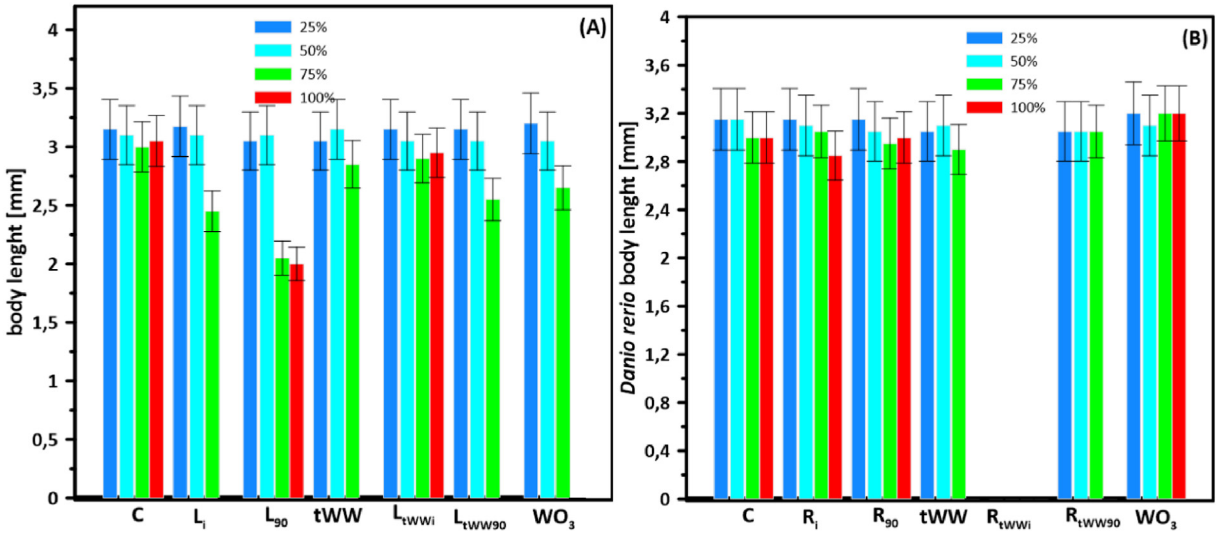

Fig. 3. Body length of *Danio rerio* in water and wastewater samples with (A) LPV and (B) RNV. C - control, tWW - treated wastewater, L_i and R_i are LPV and RNV (10 mg·L^{-1}) in distilled water, respectively; L_{90} and R_{90} are LPV and RNV in distilled water after visible light irradiation for 90 min, respectively; L_{tWWi} and R_{tWWi} are LPV and RNV (10 mg·L^{-1}) in treated wastewater, respectively; L_{tWW90} and R_{tWW90} – LPV and RNV in treated wastewater after visible light irradiation for 90 min, respectively; L_{WO3} and R_{WO3} are LPV and RNV in photocatalytically treated wastewater.

limits, the presence of traces of antiviral drugs and their intermediates can affect the survival and behavior of *Danio rerio*, putting the life of embryos at high risk (Tenorio-Chávez et al., 2020).

Understanding the mechanism of the interaction of LPV and RNT with *Danio rerio* embryos and fish is still challenging. Nevertheless, other drugs, such as naproxen, ibuprofen, and paracetamol were reported to interact with the hydrophilic groups of the membrane (Teixidó et al., 2019), affecting their development and the formation of pericardial edema and hypopigmentation. According to (Tu et al., 2016), LPV and RNT are highly protein-bound and can induce hepatotoxicity and hypersensitivity in humans. (Abou-El-Naga et al., 2020) studied the interactions of LNV/RNT with *Leishmania* spp. proteolytic activity, which led to the degenerated nuclear membrane, and the chromatin granules indicating apoptosis with accompanying acidocalcinosis were observed. On the other hand, many drugs are known as compounds causing oxidative stress and genotoxicity in aquatic organisms (Li et al., 2018; Sánchez-Aceves et al., 2021; Zheng et al., 2012), which affect the growth and development of fishes (Tenorio-Chávez et al., 2020). Simultaneously, the presence of any chemical substances, including drugs, in water and wastewater is characterized as a stressor that increases the level of serotonin in fish brains (Rothe et al., 2021).

Hashiguchi et al. (2021) noted similar observations (malformations) on the toxicity of effluents from oil palm milling, suggesting that an appropriate wastewater treatment should be designed. A 2% removal efficiency of antiviral drugs in the WWTPs was estimated by (Sanderson et al., 2004), and photolysis was demonstrated as a major path for the transformation of pharmaceuticals in the environment and water (Zhou et al., 2015). This process is generally affected by the presence of dissolved ions and dissolved organic matter (DOM) (Zhou et al., 2015). The residual concentration of LPV (Table 1) indicates that the treated wastewater containing the traces of LPV and photocatalytically treated wastewater containing the traces of LNV are very toxic ($EC_{50} < 1$ mg·L^{-1}) to aquatic organisms (Zhou et al., 2015). The toxicity may arise from the presence of traces of LNV and its oxidation products. The oxidation of LPV leads to the formation of substituted acetamide and 2,6-dimethylphenoxy acetic acid (Chitturi et al., 2008) as the products of hydrolysis and CN bond cleavage.

3.3. Microtox® studies

It can be seen that the visible light irradiation of water containing LPV caused significant changes in the response of *A. fischeri* (Fig. 4A). Before visible light irradiation of water containing LPV, the growth of bacteria was stimulated; however, the bioluminescence was inhibited up to 20% after 90 min of treatment. After the first slight inhibition of bioluminescence (after 5 min of contact), the tWW induced the habituation of bacteria to the environment (after 15 min of contact). Even at the high concentration of LPV in the tWW, some stimulation on the bacterial growth was noticed. Evidently, the effect of the wastewater matrix was observed, and the stimulation was increased (Ferreira et al., 2002). However, when the LPV-containing tWW was irradiated for 90 min, it revealed a significant toxicity as bioluminescence was hindered >40%. Again, the obtained data clearly indicate that visible light irradiation of water and wastewater samples containing LPV leads to the generation of toxic products to bacteria. This can be explained by the fact that the presence of other components in the water and wastewater matrices, such as nitrates, bicarbonates, and dissolved organic matter (DOM) affects the photo-induced processes (Kim and Tanaka, 2009; Zhou et al., 2015). Non-selective and powerful hydroxyl radicals ($^{\bullet}OH$) are created under visible light irradiation of nitrates present in water. However, $^{\bullet}OH$ can be scavenged by the reaction with bicarbonate ion, resulting in the formation of a highly selective $CO_3^{\bullet}$ (Zhou et al., 2015). DOM acts in two different ways: (i) the promotor of radicals reactions and (ii) inhibitor (radicals scavenger) (Niu et al., 2014).

RNV in water did not reveal any toxicity to *A. fischeri* (Fig. 4B). Similar to LPV, visible light irradiation affected the level of toxicity. A slight inhibition of bioluminescence was noted (up to 10%) both in water and wastewater samples although there was no RNV in the irradiated water. The decomposition products affected the bacterial activity. The bioluminescence of *A. fischeri* exposed to RNV-spiked tWW confirmed the presence of substantial toxic substances that are the most harmful to bacteria. Nevertheless, visible light irradiation of RNV-spiked tWW reduced the toxicity by 60%. This indicates that the photolysis products of RNV were less toxic. Probably, the prolongation of irradiation time beyond 90 min may further lower the toxicity to an acceptable level (20%, non-toxic solution) (Heinlaan et al., 2008). The photocatalytic treatment of RNV-spiked tWW led to the total elimination of toxicity. The data indicate that the photo-induced processes and the additional treatment of water and wastewater containing RNV (e.g., photocatalysis) enabled to minimize the toxic effects of these antiviral drugs.

4. Conclusions

In summary, the effects of two photo-induced processes (photolysis and photocatalysis) on the toxicity of treated water and wastewater containing

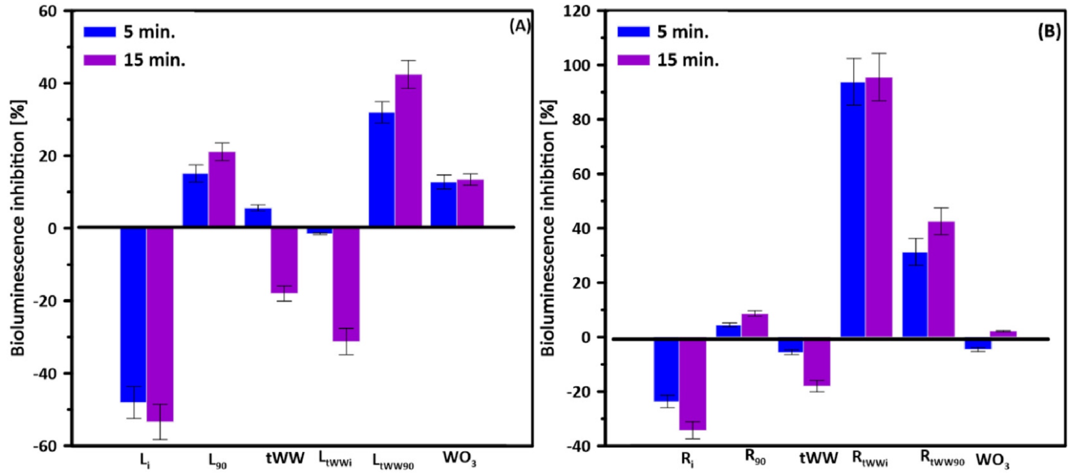

Fig. 4. Microtox® studies of water and wastewater samples with (A) LPV and (B) RNV. L_i and R_i are LPV and RNV (10 mg·L^{-1}) in distilled water, respectively; L_{90} and R_{90} are LPV and RNV in distilled water after visible light irradiation for 90 min, respectively; L_{tWWi} and R_{tWWi} are LPV and RNV (10 mg·L^{-1}) in treated wastewater, respectively; L_{tWW90} and R_{tWW90} are LPV and RNV in treated wastewater after 90 min visible light irradiation, respectively; *WO₃* is LPV or RNV in photocatalytically treated wastewater.

lopinavir and ritonavir to zebrafish (*Danio rerio*) and marine bacteria (*Allivibrio fischeri*) were studied. Both photo-induced processes influenced the survival and growth of *Danio rerio* and *Allivibrio fischeri*. The photo-induced treatment of lopinavir-containing wastewater showed a lethal effect on *Danio rerio*, and several malformations, including pericardial edema, tail autophagy, and scoliosis were observed. The non-toxic wastewater containing lopinavir after visible light irradiation for 90 min showed some toxicity, and the embryos of *Danio rerio* were shorter (23%), and scoliosis was observed. A similar trend was observed in water and wastewater samples treated by photocatalysis. The products formed during the photo-induced processes of lopinavir were toxic, causing sub-lethal changes to the tested organisms. Hence, lopinavir must be removed from water and wastewater before photo-induced processes. In contrast, the photo-induced processes of ritonavir-containing water and wastewater significantly reduced the toxicity, confirming the essential role of the photo-induced processes in minimizing the toxic effect. It was found that even though the physicochemical parameters of water and wastewater are within the standard requirements/limits, the traces of antiviral drugs and their intermediates can still affect the survival and behavior of *Danio rerio* and *Allivibrio fischeri*.

CRediT authorship contribution statement

Bożena Czech: Investigation, Methodology, Visualization, Writing-Reviewing and Editing, Conceptualization, Validation, Supervision; **Agnieszka Krzyszczak**: Investigation; **Anna Boguszewska-Czubara**: Investigation, Methodology, Visualization; **Grzegorz Opielak**: Investigation; **Izabela Jośko**: Methodology, Writing- Reviewing and Editing, Validation, Supervision; **Mirabbos Hojamberdiev**: Writing- Reviewing and Editing, Conceptualization, Validation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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