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Genotoxicity of treated wastewater disinfected with peracetic acid

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ABSTRACT

Disinfection of treated wastewater and its reuse for breeding, agricultural or recreational purposes is a beneficial solution due to water deficits in the world. The risks associated with treated wastewater disinfection include, among others, possibility of the formation of harmful by-products (DBPs). Unmonitored presence of DBPs in treated wastewater is potentially harmful to organisms in ecosystems and to human health. The aim of the research was to compare the genotoxicity of treated wastewater from full-scale municipal wastewater treatment plant before and after the disinfection process with peracetic acid with parameters ensuring satisfactory inactivation of microorganisms. Genotoxicity to *Escherichia coli* bacteria was tested with SOS Chromotest. The influence on the genetic material of *Daphnia magna* crustaceans was determined by the random amplification of polymorphic DNA – polymerase chain reaction (RAPD-PCR). Treated wastewater before disinfection showed genotoxicity to *E. coli* and *D. magna*. However, although treated wastewater disinfected with peracetic acid turned out to loose genotoxic potential to bacteria, it generated changes in the genetic material of crustaceans exposed to wastewater after the disinfection process. Significant decrease in the genetic stability of DNA in RAPD-PCR was observed, which increased with the extension of the disinfection time and the increasing concentration of the disinfectant. The conducted research confirms the genotoxic potential of treated wastewater before and after disinfection with peracetic acid. Therefore, it may pose a threat to organisms of aquatic ecosystems when discharged into receiving reservoirs and reused in the event of a water shortage.

Keywords: Peracetic acid; Treated wastewater disinfection; Treated wastewater genotoxicity

1. Introduction

Treated wastewater contains a variety of pathogens and drug-resistant microorganisms, which are capable of survival in the environment long enough to alter changes in ecosystems or to be transmitted to humans [1,2]. Disinfection of effluents from wastewater treatment plants (WWTPs) can significantly reduce the abundance of pathogens and thus decrease the risk of disease and drug-resistance transmission [3].

Among many existing disinfection strategies there are some widely recognized, like chlorination, ultraviolet irradiation (UV), ozonation and membrane filtration, for which data regarding installation, maintenance costs and life cycles of the systems are already available [4]. There are also some disinfection techniques which are now being introduced and first installations are tested on full-scale WWTPs effluents [5]. Application of peracetic acid (CH_3COOH, PAA) is one of the most considered due to a wide spectrum of antimicrobial activity manifested by this disinfectant and reported limited regrowth of coliforms [6–8]. PAA is available commercially as an acidic quaternary equilibrium mixture of peracetic acid, hydrogen peroxide, acetic acid, and

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water. Acetic acid is biodegraded to carbon dioxide and hydrogen peroxide degrades to oxygen and water, neither of which is considered toxic to aquatic life. However, as hydrogen peroxide remains as a product in the treated water, its toxicity to organisms in the receiving waters needs consideration [9]. Due to its strong oxidizing power some authors raise doubts of safe use of PAA as ecologically friendly disinfectant for wastewater discharges [10–13].

An unplanned effect of disinfection may be the reaction of disinfectants with anthropogenic contaminants, bromide/iodide or natural organic matter to form disinfection by-products (DBPs) [11,12,14,15]. Ecotoxicity of some DBPs may be observed by conventional assays, but molecular testing methods seem to be much more relevant tools. It is because interaction of DBPs dissolved in wastewater with the genetic material of aquatic organisms can alter proper protein production and cellular function and trigger changes involving one generation (e.g., cancer, embryotoxicity) or, particularly in the case of constant exposition, affect populations by, for example, hereditary effects, fertility reduction, reproductive loss. Following decline in genetic diversity, causes inability to adapt to changing environmental conditions and ecosystem imbalance. Emerging DBPs present constantly in the environment may also pose hazard to people's health (including cancerogenesis). Some of them proved to be cytotoxic, neurotoxic, mutagenic, genotoxic, carcinogenic and teratogenic [1,2,14,16]. Therefore, data concerning mutagenicity potential of treated wastewater and exposure of organisms to genotoxic DBPs are vitally important [17,18]. Among many molecular tests detecting different changes in the genetic material and involving different aquatic species, SOS Chromotest and RAPD-PCR fingerprinting seem to constitute suitable test battery for genotoxicity assessment of treated wastewater before and after disinfection.

The sensitive quantitative colorimetric SOS Chromotest consists in induction (by genotoxic compounds) of bacterial (*Escherichia coli*, genetically engineered mutant strain) SOS repair system associated with a gene responsible for the synthesis of β-galactosidase and measurement of its enzymatic activity [19]. When DNA damage occurs, the SOS system is activated and β-galactosidase gets transcribed proportionally to the level of SOS induction. The strain of *E. coli* was made constitutive for the alkaline phosphatase synthesis to verify if general protein synthesis is stable, which makes it possible to simultaneously check the cytotoxicity of the tested mixtures via alkaline phosphatase activity measurement. SOS Chromotest may be used for direct genotoxins, but also indirect genotoxins may be detected through the use of S9 liver homogenate from Aroclor 1254-induced rats. For this reason, procarcinogens which require metabolic conversion to reactive metabolites before they interact with DNA, may also be identified [11,19].

RAPD-PCR (random amplified polymorphic DNA – polymerase chain reaction) is one of the useful methods for assessing DNA variations. The assay allows rapid analysis of a large number of samples and is suitable for any extracted DNA of sufficient quality [20–22]. Genome sequence in bioindicators are not needed because arbitrary primers are used. Changes in DNA fingerprint (electrophoretic patterns in the compared samples) indicate DNA alterations from point mutations to complex chromosomal rearrangements [20].

The main goal of this research was to determine the consequences of disinfection of effluents from full-scale WWTP using peracetic acid, which may increase their genotoxicity to organisms. The research deals with ambiguous reports considering genotoxicity appearance after PAA disinfection [13]. As far as authors are concerned, this work is the first to assess genotoxicity to *Daphnia magna* (representative bioindicator of fresh waters) of treated wastewater after disinfection using RAPD fingerprinting. The novelty of the study is based also on disinfection of real treated wastewater from full-scale municipal WWTPs, as most research in this field is carried out using synthetic wastewater [9,12,23,24]. As shown by preliminary studies [23], in real treated wastewater the susceptibility of microorganisms to inactivation may significantly change and micropollutants and contaminants of emerging concern (CECs), including pharmaceuticals, personal care products, synthetic hormones, and endocrine-disrupting chemicals (EDCs) may interact with disinfectants to form DBPs [25–27].

2. Materials and methods

2.1. Preliminary studies: selection of disinfection parameters

The final nominal concentration of PAA and set contact time presented in the study were carefully selected based on the dose range commonly used in WWTPs [28,29] and the results of preliminary tests designed to achieve satisfactory bacterial inactivation level on samples of treated wastewater collected 5 times in different seasons and from different wastewater treatment plants (located in Warsaw and Stare Babice, Poland). In the preliminary tests, the parameters for each disinfection process were: time (20 min, 60 min) and disinfectant dose (0.6, 2, 3, 5, 10, and 15 mg/L) and total suspended solids varied from 5.9 to 30 mg/L depending on sampling time and location.

2.2. Samples of treated wastewater

The samples were collected between January 2021 and March 2022 from the largest municipal WWTP in Poland ("Czajka", Warsaw). The mechanical–biological WWTP receives typical municipal wastewater and has capacity of 435,300 m^3/d. After finishing of biological wastewater treatment processes, the mixture of wastewater with activated sludge flows from a single bioreactor to two radial secondary settling tanks. Treated wastewater from the secondary sedimentation tanks is discharged to the drainage channel. During low water levels (≤77.92 m above sea level), the treated wastewater flows through the treated sewage channel, and then by gravity, through the flow meter chamber and the drain chamber, is released through the discharge channels into Vistula River. In the event of an increased water level in the Vistula River and an increased flow of wastewater, a pumping station is started up. All the above-presented data was provided by the plant operator.

Samples of treated wastewater were delivered to the laboratory within 1 h. The samples of effluent, subjected to disinfection, were characterized, by parameters presented in Table 1.

2.3. Microbiological analysis

Enumeration of culturable psychrophilic and mesophilic bacteria, as well as *E. coli*, *Clostridium perfringens* and *Enterococcus faecalis* was performed in accordance with PN-EN ISO 9308-3, PN-EN ISO 14189:2016-10 and PN-EN ISO 7899-1, respectively [30–32]. Additionally, the assessment of total live biomass in treated wastewater before and after the disinfection processes, was carried out based on ATP determination in accordance with DeltaTox ATP manual (Modern Water, UK). Each bacterial enumeration and ATP determination was done in triplicate.

2.4. Disinfectant preparation

PAA solution (CAS no: 79-21-0) containing 38%–40% w/w of technical grade disinfectant was purchased from Sigma-Aldrich (Poznań, Poland). From this solution, a working solution of 1 g/L PAA was made, which was quantified and analyzed using titration (iodometric method).

2.5. Peracetic acid disinfection

For the disinfection fresh effluent was used, which was collected in the morning of the same day. The experiment was carried out in 1-L glass vessels, the contents of which was slowly stirred on magnetic stirrers. Based on results from the microbiological analysis the nominal test concentrations of 0.6, 2 and 5 mg/L were selected and the appropriate volume of peracetic acid was added from previously prepared working solution. The disinfection lasted 20 min and the reaction was stopped by adding a sterile 0.2 N sodium thiosulphate solution stoichiometrically. Finally, each disinfection process was conducted in at least three repetitions.

3. Genotoxicity tests

3.1. SOS Chromotest

The colorimetric assay employing mutant strains PQ37 of *E. coli* was conducted following the methodology described in the implementing instructions of Environmental Bio-Detection Products Inc. [33]. The quantitative assay was conducted in versions with and without metabolic activation (S9 fraction) with application of two standard genotoxic solutions: 4NQO (4-nitro-quinoline-1-oxide) and 2AA (2-amino-anthracene). S9 mix contained S9 extract of Aroclor-induced rat liver and was prepared according to kit instructions [33]. The bacteria were grown at a stable temperature of 37°C in a temperature-controlled incubator (Biogenet, Józefów, Polska). 10 μL aliquots of sample, standards dilutions and controls were dispensed into appropriate wells on the 96-well microplate. Subsequently, 100 μL of the bacterial suspension (diluted overnight culture) or 100 μL of bacterial suspension with S9 were added to all designated wells and the microplates were incubated at 37°C for 2 h. Alkaline phosphatase chromogen was then added and the mix was incubated at 37°C for 60 min until green colour developed. The MB100-4A THERMO-SHAKER for microplates with rotable platform (Hangzhou Allsheng Instruments Co., Ltd., China) was used for the development of the enzymatic activities. The enzymatic activity was measured by photometric measurement at 620 nm (genotoxic activity) and 405 nm (viability) using LT-4500 microplate reader (Beijing LabTech Instruments Co., Ltd., China). Readings were taken immediately after the colorimetric incubation time.

Genotoxicity assessment was based on the SOS induction factors (SOSIF) and were calculated according to the formula:

$$\mathrm{SOSIF} = \frac{\left(\dfrac{A_{620}S - A_{620}B}{A_{620}N - A_{620}B}\right)}{\left(\dfrac{A_{405}S - A_{405}B}{A_{405}N - A_{405}B}\right)} \quad (1)$$

where $A_{620}S$ – absorbance readings at λ = 620 nm for sample wells; $A_{405}S$ – absorbance readings at λ = 405 nm for sample wells; $A_{620}N$ – absorbance readings at λ = 620 nm for negative control wells; $A_{405}N$ – absorbance readings at λ = 405 nm for negative control wells; $A_{620}B$ – averaged absorbance readings at λ = 620 nm for reagent blank wells; $A_{620}N$ – averaged absorbance readings at λ = 405 nm for reagent blank wells.

Genotoxicity assessment was performed according to the criteria presented in Table 2 followed by some previously published studies [34,35].

Cytotoxicity assessment was based on survival rate (SR, %), which were calculated according to formula:

$$\mathrm{SR} = \frac{A_{405}S}{A_{405}N} \times 100\% \quad (2)$$

Table 1
Chemical characteristics of treated wastewater of "Czajka" WWTP in the period of January 2021–March 2022

Parameters (mg/L)	Method	Average value (±standard deviation)
pH	PN-EN ISO 10523:2012	7.65 (±0.15)
Suspended solids	PN-EN 872:2007	6.42 (±3.25)
COD	PN-ISO 15705:2005	30.39 (±6.34)
BOD	PN-EN ISO 5815-1:2019	3.53 (±0.40)
Nitrogen	PB-ZLA-OC-17	8.57 (±1.19)
Ammonia	PN-ISO 5664:2002	1.96 (±0.50)
Nitrite	PB-ZLA-OC-09	0.09 (±0.03)
Nitrate	PB-ZLA-OC-25	4.79 (±1.22)
Total phosphorus	PB-ZLA-OC-12	0.28 (±0.11)
Phosphates	PB-ZLA-OC-07	0.14 (±0.08)

Table 2
Classification of genotoxic intensity according to significance level of the response

SOSIF < 1.5	No genotoxicity	–
1.5 ≤ SOSIF < 2.0	Slight genotoxicity	+
2.0 ≤ SOSIF < 5.0	Moderate genotoxicity	++
SOSIF ≥ 5.0	Strong genotoxicity	+++

A survival rate of 80% was required to confirm a positive result of genotoxicity.

3.2. Generation of D. magna DNA profiles using RAPD-PCR

3.2.1. Culture of D. magna and exposition to wastewater samples

D. magna (Straus, 1820) came from the own laboratory culture of Department of Biology, Faculty of Building Services, Hydro and Environmental Engineering, Warsaw University of Technology. The animals were maintained in 15 L aquariums in aquarium water mixed (1:1) with OECD medium [36] at a temperature of 20°C–22°C with a photoperiod of 16 h light (4,000 lux): 8 h dark. The medium was changed once every week. Animals were fed daily with *Chlorella vulgaris* algae (1.5–2.5 × 10^8 cells/d) alternating with dried *C. vulgaris* (Purella, Superfoods, Warsaw, Poland).

Freshly born neonates (less than 48 h) were exposed in replicate groups of 20 to treated wastewater samples (before disinfection, disinfected with 0.6, 2 and 5 mg/L PAA) and the controls (negative – medium, positive – 3% H_2O_2). After 48 h of incubation with treated wastewater samples animals were collected for DNA extraction.

3.2.2. RAPD fingerprinting

All RAPD-PCR chemicals were purchased from A&A Biotechnology (Gdańsk, Poland). Total DNA from *D. magna* was extracted and purified using DNA-Xpure™ Cell micro using the protocol supplied by the manufacturer. The DNA profiles of *D. magna* were generated in RAPD-PCR reactions performed in a reaction volume of 25 μL. The decamer oligonucleotides (primers) OPB7 (GGTGACGCAG), OPB8 (GTCCACACGG), OPA9 (GGGTAACGCC), OPB10 (CTGCTGGGAC), were obtained from Environmental Laboratory of DNA Sequencing and Synthesis IBB PAS (Warsaw, Poland). One of the primers was used for each amplification. Approximately 25 ng of *D. magna* genomic DNA was subjected to RAPD amplification with reaction mixtures containing PCR Mix (0.1 U/μL Taq DNA polymerase, 4 mM, $MgCl_2$, 1xPCR Buffer, 0.5 mM of each dNTP) (A&A Biotechnology, Poland) and a primer concentration of 10 μM. Amplifications were performed in DNA thermocycler (Mastercycler pro, Eppendorf) programmed for 4 min at 95°C (initial denaturation), 39 consecutive cycles each consisting of 1 min at 95°C (denaturation), 1 min at 40°C (annealing), 1 min at 74°C (extension), and followed by 1 cycle for 10 min at 74°C (final extension). Control PCRs lacking genomic DNA were run with every set of samples. Reaction mixtures were kept in 4°C prior to use.

3.2.3. Electrophoresis and analysis of DNA profiles

RAPD reaction products (7–8 μL) were separated by electrophoresis on 1% w/v agarose gels in 1xTris-Borate-EDTA buffer (40 mM Tris base, 20 mM boric and 1 mM EDTA) at room temperature at 80–100 V (297 mA) for about 30–40 min in SUBDNA apparatus (Kucharczyk, Poland). GeneRuler 1 kb DNA Ladder (Fermentas, USA) (7 μL) was used as the molecular weight DNA standard. DNA bands were stained with ethidium bromide (0.5 mg/mL, 10 min), rinsed with tap water (10 min), visualized and photographed under UV light. The size of each amplification product was automatically estimated using the GelDoc-It Imaging System (Ultra-Violet Products Ltd, USA). Digital processing of the images and computational analysis was performed using Gelix One 1-D Analysis Software (Biostep, Germany). All amplifications were repeated twice in order to confirm there producibility of RAPD patterns. Only repeatable and clear amplification bands were scored for the construction of the data matrix.

3.2.4. Data analysis

Polymorphism observed in RAPD profiles included disappearance of normal amplification products (bands) and occurrence of new bands in comparison with control RAPD profiles (negative and positive) [20]. Influence of treated wastewater before and after disinfection on the genetic material of *D. magna* was assessed on the basis of genetic similarity index of bands' profiles (S, %) and genetic stability of DNA (GTS, %) between tested and control samples for individual primers. S was calculated as the proportion of amplification products which were not polymorphic with respect to the total number of amplified products, 2 × number of shared fragments/total number of fragments [37]. GTS was calculated for each primer as 100 – (100a/n), where a was the average number of changes in DNA profiles of each sample tested and n the total number of bands in the control DNA profiles. Mean values and standard deviations were calculated. Changes in these values were calculated as a percentage of the negative control (set to 100%) and allowed to distinguish organisms exposed to genotoxic agents.

4. Results and discussion

The recognition of ecologically friendly techniques of wastewater disinfection could be one of the most significant advances in the field of environmental engineering. Considering public health as an ultimate aim, and taking into account diminishing resources of potable water worldwide, disinfection of treated wastewater will soon become a necessary process prior to discharging it into environment. PAA seems to be more environmentally friendly disinfectant compared to other conventional chemicals used. Although, formation of DBPs is reported, they have mostly been recognized to be harmless [7,11,38,39]. The most prominent PAA's DBPs are carboxylic acids, low concentrations of aldehydes and ketones, with limited or undetectable amounts of halogenated DBPs (e.g., haloacetic acids (HAA) or trihalomethanes (THMs)). Theoretically, brominated phenol by-products formation could potentially occur, but laboratory studies have shown that the brominated phenol formation can only be achieved under unrealistic wastewater treatment conditions (pH 3.8–4.2) with high concentrations of PAA [11,13]. However, high levels of I-HAAs and I-THMs were observed by Xue et al. [13] after disinfection by PAA solution with higher PAA than H_2O_2 which was connected with high levels of I in disinfected water. Iodinated DBPs may pose hazard to environment because they are generally more toxic than their brominated and chlorinated analogs [40]. Still, PAA remains a strong oxidizing agent with lower implementation and operation costs and lower corrosive properties compared to other existing technologies and further studies are

needed to confirm or deny its safe use in wastewater treatment [39,41,42].

Therefore, the primary aim of this work was to examine the genotoxicity of the effluents from the full-scale municipal WWTP, subjected to PAA disinfection, in order to predict the ecotoxicological consequences of disinfected wastewater discharges into aquatic ecosystems.

Before adopting a particular disinfection system it is advisable to monitor wastewater mutagenicity at different times of the year at various dosages of disinfectant keeping in mind that microbial contamination of surface water should still be considered the main public health problem in wastewater management [38]. The results of preliminary studies (data not shown) on treated wastewater samples collected in different seasons over 14 months confirmed the observations of Rossi et al. [8], that disinfectant active concentration was the main factor affecting log-survival ratios, while the effect of contact time was lower after 20 min of disinfection. Also increasing the nominal concentration of PAA above 5 mg/L did not significantly increase the inactivation of microorganisms.

Selected parameters of disinfection (20 min: 0.6, 2, 5 mg/L) showed satisfactory bacterial inactivation 72%–100% (0.5–2.5 log) (Table 3). Mesophilic bacteria and *C. perfringens* turned out to be the most resistant, however 5 mg/L PAA was surely enough to inactivate almost 100% of culturable bacteria in treated wastewater. Similar disinfection parameters (5 mg/L PAA, 20 min) were used by da Costa et al. [1] for treating effluents from Brazilian WWTP in rainy season. They noticed 4.1 log removal (reduction from 10.12×10^5 to 84 NPM/100 mL) of *E. coli* and 3.4 log removal for total coliforms. In our work *E. coli* and *E. faecalis* counts were below detection level, with 100% inactivation out of 1.8×10^3 and 11.2 CFU/mL, respectively. In shorter contact time (10 min), in concentrations ranging from 2.5 to 30 mg/L PAA, Chhetri et al. [9] observed disinfection efficiency for *E. coli* in range of 3.4–5.6 log units, but the samples for treatment came from simulated extended overflow containing 5% of raw wastewater resulting in much higher bacterial counts before disinfection. Additionally, contrary to the reports of these authors, we did not notice weak removal of *E. faecalis*.

Much higher abundance of microorganisms in tested samples was determined by the sensitive DeltaTox ATP test (Table 4). Standard colony count or even microplate methods did not detect all viable cells present in the samples (ratio CFU/ME did not exceed 1%) [43]. The ATP analysis showed that the percentage inactivation of viable culturable and unculturable bacteria was lower (23%–96%), resulting in 1.02–1.23 log inactivation after disinfection using described parameters.

In the present study, the SOS Chromotest was performed, with and without metabolic activation. Such approach expands the detection capabilities of the assay, since many carcinogens are known to require metabolic conversion (biotransformation) to reactive metabolites before they interact with DNA. Undiluted and diluted 2–4x treated wastewater, not subjected to disinfection, showed genotoxicity to bioindicators (Table 5). This stands in line with observation of de Souza Celente et al. [17] who also noticed that WWTP's treated effluent presented genotoxicity detected by comet assay. Interestingly, the same samples of treated wastewater were not genotoxic to bacteria when metabolic activation was introduced. This means that biotransformation of mutagens must have occurred to less genotoxic compounds. Obtained SOSIF values for treated wastewater after disinfection suggest that the potential of genotoxicity of treated wastewater

Table 3
Number of culturable bacteria (CFU/mL) in treated wastewater before and after disinfection by PAA (0.6, 2 and 5 mg/L; 20 min). Inactivation (in percentage and log) is presented in parenthesis for each disinfection method

Bacteria	Before disinfection ± SD	PAA 0.6 mg/L	PAA 2 mg/L	PAA 5 mg/L
Psychrophilic	$6.9 \times 10^4 \pm 7.9 \times 10^4$	5.4×10^3 (92; 1.1)	1.6×10^3 (98; 1.6)	2.4×10^2 (100; 2.5)
Mesophilic	$7.4 \times 10^3 \pm 2.3 \times 10^3$	2.1×10^3 (72; 0.5)	2.1×10^3 (72; 0.5)	7.4×10^1 (99; 2.0)
Escherichia coli	$1.8 \times 10^3 \pm 1.8 \times 10^3$	3.5×10^2 (80; 0.7)	3.9×10^1 (98; 1.7)	n.d.[a] (100; n.a.[b])
Clostridium perfringens	5.3 ± 5.6	1.4 (74; 0.6)	0.4 (92; 1.1)	0.2 (97; 1.5)
Enterococcus faecalis	11.2 ± 12.4	0.9 (92; 1.1)	1 (95; 1.3)	n.d.[a] (100; n.a.[b])

[a]n.d. – not detected;
[b]n.a. – not available.

Table 4
Total ATP (tATP), free-available ATP (fATP), intracellular ATP (cATP), reflecting total live biomass (both culturable and unculturable), inactivation (percentage and log in parenthesis) and percentage of bacterial cells detected by the colony count method (CFU/ME) in treated wastewater during disinfection process

PAA concentration (mg/L)	tATP (ME/mL)	cATP (ME/mL)	fATP (ME/mL)	CFU/ME (%)
0	9.9×10^6	9.7×10^6	5.7×10^4	0.15
0.6	7.9×10^6	7.5×10^6 (23%; 1.02)	4×10^5	0.11
2	9.5×10^5	8.4×10^5 (89%; 1.15)	9.1×10^4	0.34
5	5×10^5	4.4×10^5 (96%; 1.23)	3.4×10^4	0.07

ME – microbial equivalents; tATP – total ATP; cATP – intracellular ATP; fATP – free-available ATP.

Table 5
Results of SOS Chromotest on treated wastewater before and after disinfection with PAA (SOSIF – SOS induction factor; SD – standard deviation, SR – survival rate)

Sample		Concentration (%)	SOSIF ± SD	SR (%)	Genotoxicity assessment
Before disinfection	without S9	100	**2.37** ± 0.44	≥90.33	++
		50	**2.37** ± 0.84		++
		25	**2.53** ± 0.72		++
		12.5	**1.71** ± 1.39		+
	with S9	100	0.82 ± 0.12	≥91.04	–
		50	0.97 ± 0.40		–
		25	0.64 ± 0.22		–
		12.5	0.77 ± 0.24		–
After disinfection with 0.6 mg/L PAA	without S9	100	**2.07** ± 0.32	≥89.88	++
		50	1.38 ± 0.24		–
		25	1.13 ± 0.21		–
		12.5	0.97 ± 0.35		–
	with S9	100	0.82 ± 0.12	≥91.01	–
		50	0.97 ± 0.40		–
		25	0.64 ± 0.22		–
		12.5	0.77 ± 0.24		–
After disinfection with 2 mg/L PAA	without S9	100	**1.84** ± 0.73	≥93.93	+
		50	1.02 ± 0.34		–
		25	1.37 ± 0.25		–
		12.5	0.82 ± 0.15		–
	with S9	100	0.69 ± 0.18	≥93.14	–
		50	0.74 ± 0.26		–
		25	0.78 ± 0.07		–
		12.5	0.81 ± 0.20		–
After disinfection with 5 mg/L PAA	without S9	100	1.16 ± 0.05	≥96.48	–
		50	1.10 ± 0.01		–
		25	0.92 ± 0.04		–
		12.5	0.99 ± 0.17		–
	with S9	100	0.75 ± 0.22	≥93.04	–
		50	0.50 ± 0.13		–
		25	0.74 ± 0.14		–
		12.5	0.76 ± 0.06		–

Genotoxic samples are indicated in bold letters.

declined after disinfection with PAA with the complete loss of genotoxicity after disinfection with 5 mg/L PAA. It can therefore be concluded that treated wastewater would be unlikely genotoxic to mammals. Also recently, Bhuvaneshwari et al. [44] reported that ozonation, based like PAA application on oxidizing power, could degrade genotoxic compounds in some effluents. Promising results were also presented by Maurício et al. [45] who observed PAA's potential for estradiol removal, contradicting other reports on not enough power for estrone oxidation [46]. Both organic compounds are known as EDC which can interfere with endocrine system and trigger hormonal changes in organisms present in aquatic environment.

Several qualitative changes in the RAPD pattern were observed while using different primers (Fig. 1) for all treatments compared to the control.

Genetic material of bioindicators exposed to water (Cw) and treated wastewater before disinfection (Cn) varied consistently (Table 6). Genetic stability (GTS) equalled on average 4.7% for treated wastewater and –18.2% for positive control in relation to Cw, which reflects the difference by 95.3% and 118.2%, respectively. The degree of genetic similarity (S) of band profiles to the control showed that treated wastewater triggered changes in genetic material of bioindicators (Table 7). S value for disinfected effluents in relation to treated wastewater before disinfection (Cn) varied from 0 to 57.1% for all primers and was the lower, the higher was the PAA concentration used for disinfection, regardless of the primer used. This means that genetic material of daphnids exposed to treated wastewater disinfected with lower concentrations of PAA was more similar to the genetic material of daphnids exposed to Cn. Disinfection of treated wastewater with

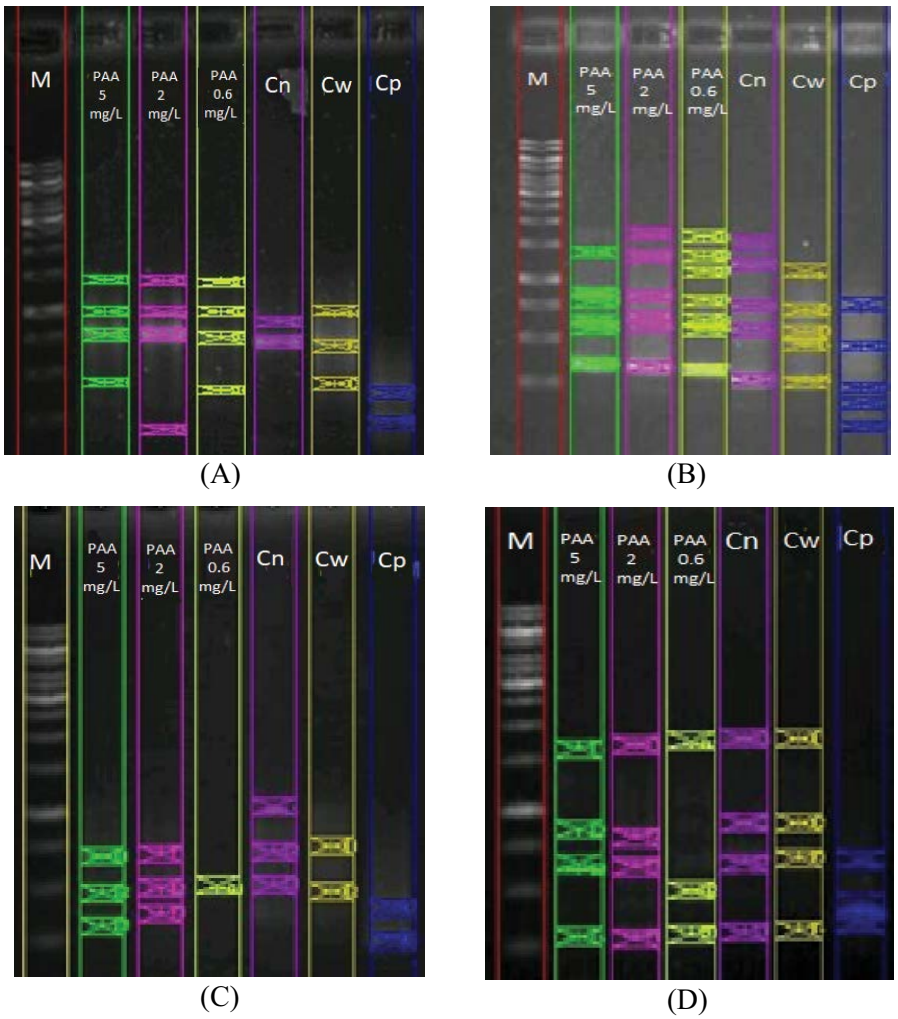

Fig. 1. Similarity diagram for DNA fingerprints generated with primers: (A) OPB7, (B) OPB8, (C) OPA9 and (D) OPB10 for *Daphnia magna* incubated in treated wastewater before (Cn) and after disinfection with PAA (0.6, 2 and 5 mg/L), H_2O (Cw – negative control) and H_2O_2 (positive control, 3%) (Vision Works L.S.M. program).

Table 6
Genetic stability (GTS) of DNA of *Daphnia magna* incubated in treated wastewater (Cn) and positive control (Cp) in relation to Cw (water) – set to 100% for primers OPB7, OPB8, OPA9 and OPB10

Primer	Treated wastewater (Cn)	Positive control (Cp)
OPB7	–50.0	–44.4
OPB8	13.3	6.74
OPA9	33.3	–27.8
OPB10	22.2	–7.4
Average	4.7	–18.2

5 mg/L PAA gave values equal to 0% or low values in all variants. Total DNA od *D. magna* after 48 h of exposition to disinfected effluents differed also from the genetic material of crustaceans exposed to water (Cw). S values ranged from 0% to 50% also with negative correlation with concentration of PAA, except from OPB7 primer, for which S was consistently equal 33.3%. The lowest values of S were observed in relation to positive control (crustaceans exposed to 3% H_2O_2; Cp) and ranged from 0% to 40%. Also, the higher was the concentration of PAA used for disinfection, the higher was the similarity of tested samples to the positive control.

Average value of genetic stability (GTS) in tested samples (DNA from *D. magna* exposed to disinfected effluents) differed significantly from GTS of negative and positive controls (Table 7). Samples isolated from crustaceans exposed to treated wastewater disinfected for 20 min with 5 mg/L PAA showed lowest average GTS in relation to Cw, equal 1.25% (difference from the negative control by 98.75%). The highest value was obtained for genetic material of daphnids exposed to treated wastewater disinfected for 20 min with 0.6 mg/L PAA and equalled on average to 45.8% (difference from the negative control by 54.2%).

Both short-term bioassays performed on bacteria and crustaceans showed a consistent presence of genotoxic effects of treated wastewater before disinfection, which most probably can be controlled by dilution as suggested by Chhetri et al. [40] for dealing with ecotoxicity of combined sewer

Table 7
Values of the degree of genetic similarity (S) and genetic stability (GTS) of the tested samples containing the genetic material of *Daphnia magna* incubated in treated wastewater disinfected PAA in concentrations of 0.6, 2 and 5 mg/L for 20 min in relation to treated wastewater (Cn), water (Cw) and positive control (Cp)

Primer	PAA concentration (mg/L)	S relative to Cn (%)	S relative to Cw (%)	S relative to Cp (%)	GTS relative to Cw (%)	GTS relative to Cn (%)	GTS relative to Cp (%)
	0.6	0.0	33.3	0.0	33.3	–100.0	–50.0
OPB7	2	0.0	33.3	0.0	0.0	–100.0	–100.0
	5	0.0	33.3	33.3	0.0	–100.0	–100.0
	0.6	36.4	33.3	16.7	0.0	20.0	0.0
OPB8	2	18.2	18.2	18.2	0.0	–20.0	–20.0
	5	16.7	0.0	0.0	–20.0	–20.0	–20.0
	0.6	50.0	40.0	40.0	100.0	100.0	50.0
OPA9	2	33.3	33.3	0.0	0.0	33.3	0.0
	5	0.0	0.0	0.0	0.0	0.0	–50.0
	0.6	57.1	50.0	28.6	50.0	100.0	0.0
OPB10	2	25.0	28.6	28.6	50.0	25.0	0.0
	5	0.0	25.0	0.0	25.0	0.0	0.0

overflows. However, the study using *Tradescantia* sp. plants revealed genotoxicity of water courses downstream WWTP in micronucleus assay suggesting that discharges of wastewater treatment effluents reduced water quality at all examined sites in Brazil [41]. It is therefore justified to monitor the waters downstream of the WWTPs outflows.

In the present study disinfection with PAA lowered genotoxicity in SOS Chromotest and increased it in RAPD assay. Ambiguity and contradictions are present also in the research of other authors on treated wastewater and treated wastewater after PAA disinfection ecotoxicity and genotoxicity, suggesting no ecotoxicity/ecotoxicity [16], toxicity to *Raphidocelis subcapitata* algae [42], borderline genotoxicity in *Salmonella typhimurium* and *Tradescantia* micronucleus test and not elevated genotoxicity after PAA disinfection [10]. There is also a reasonable doubt among researchers that ecotoxic or genotoxic effects on aquatic biota downstream of a WWTP does not only depend on the wastewater treatment and disinfection technology, but to a high extent also on the composition of the raw wastewater (seasonally variable), presence of DBPs precursors and the surface water quality upstream of the WWTP [38,39].

This investigation showed that the use of the applied disinfectants in wastewater treatment should be reviewed, because under the experimental conditions tested, PAA known as ecotoxicologically safe, was capable of producing genotoxicity in treated wastewater on one of the tested species. The present study emphasized also the importance of using a battery of genotoxicity tests with representative organisms of different biological structures in order to assess hazards of releasing disinfected wastewaters into aquatic environments.

5. Conclusions

This study demonstrated the possible concerns of using PAA as an alternative disinfectant during municipal wastewater treatment. Summing up, there are a number of observations/insights which emerge from this work: (1) Bacteria present in treated wastewater are sensitive to PAA disinfection with 5 mg/L inactivating over 96% of living biomass (both culturable and non-culturable); (2) Treated wastewater was moderately genotoxic to *E. coli*. However, disinfection with PAA caused decrease in genotoxicity; (3) Addition of S9 fraction resulted in the reduction of genotoxicity, which is probably connected with biotransformation of genotoxins; (4) Treated wastewater disinfected with PAA triggered changes in the genetic material of *D. magna* – the obtained band profiles differed from those in the controls – appearance or disappearance of PCR products occurred; (5) Changes in the genetic material of *D. magna* were higher with increasing concentration of PAA used for disinfection.

Under the experimental conditions, disinfection of treated wastewater with PAA was capable of producing genotoxic effects on bioindicators, which clearly suggests potential hazard associated with disinfection of treated municipal wastewater. Biodiversity of aquatic biota cannot be protected without optimization of disinfection strategies to minimize DBPs formation and monitoring of genotoxicity of effluents disinfected by PAA. Further studies should concentrate on detecting and eliminating toxic DBPs in order to safely introduce disinfection to wastewater treatment procedures.

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Authors' contributions

Conceptualization, K.A. and N.D.; methodology, N.D. and K.A.; validation, M.Z.-R.; investigation, K.A. and N.D.; resources, K.A.; data curation, K.A.; writing—original draft

preparation, K.A.; writing—review and editing, K.A. and M.Z.-R.; visualization, K.A.; supervision, M.Z.-R.; project administration, K.A.; funding acquisition, K.A. and N.D. All authors have read and agreed to the published version of the manuscript.

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