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The peroxidase toxicity assay for the rapid evaluation of municipal effluent quality

Chantale André, Shirley-Ann Smyth, Francois Gagné

Aquatic Contaminants research division, Environment and Climate Change Canada, Montréal H2Y 2E7, Québec, Canada.

Correspondence to: Francois Gagné, Aquatic Contaminants research division, Environment and Climate Change Canada, 105 McGill, Montréal H2Y 2E7, Québec, Canada. E-mail: francois.gagne@ec.gc.ca

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Abstract

Rapid and cost-effective tests for the evaluation of industrial and municipal effluents are urgently needed for environmental monitoring. In this context, peroxidase (PER) activity has been proposed as an early-warning biosensor for assessing the water quality of various wastewater discharges and leachates. The peroxidase-toxicity (Perotox) assay includes 0.1 μg/mL PER, albumin, DNA (for the DNA protection index), 0.001% monounsaturated Tween-80, and the substrates luminol and H_2O_2. The results revealed that an initial burst of luminescence was followed by a steady decrease in luminescence within the first minute, accompanied by periodic (cyclic) changes in the intermediate compound III (CIII) of PER. When urban effluents were added, PER activity was inhibited, with a concomitant increase in lipid peroxidation, indicating oxidative damage. The reduction in PER activity was also associated with the collapse in the periodic formation of CIII, alongside a steady increase in CIII over time. The addition of DNA to the reaction mixture helped mitigate the inhibition of PER by certain effluents, enabling the calculation of a DNA protection index. The levels of polystyrene (PS) in the organic fraction of the effluents were higher in the primary aeration lagoon (36 μg/L) compared to secondary lagoons and membrane filtration (< 16 μg/L). Data analysis revealed that PER activity was negatively correlated with population size (r = -0.34) and the levels of PS materials (r = -0.56). In conclusion, the Perotox assay is proposed as a rapid screening tool for identifying potentially toxic environmental complex mixtures, such as municipal effluents.

Keywords: Peroxidase, DNA interaction, screening, wastewaters, alternative methods

INTRODUCTION

In aquatic ecosystems, urban pollution encompasses wastewater from solid waste leachates, and domestic (e.g., hospitals) and industrial activities. Additionally, rainfalls collected by sewer systems carry contaminants from sources such as tires, asphalt, and erosion from infrastructure. These contaminants may enter the sewer drainage systems, where they sometimes mix with raw wastewater before reaching treatment plants[1]. When rainfall events become more frequent and intense due to global warming, wastewater may be directly discharged into temporary holding ponds or released into receiving waters to avoid overwhelming the capacity of wastewater treatment plants[2]. Besides the usual contaminants found in municipal wastewater, such as metals, polyaromatic hydrocarbons (PAHs), detergents, and surfactants, recent studies have identified the presence of emerging pollutants, including products derived from nanotechnology, pharmaceuticals, flame retardants, and plastics[3,4]. Plastics, in particular, pose a growing challenge, as they are ubiquitous in the form of microplastics (ranging from 5 mm to 1 μm) and nanoplastics (1-1,000 nm)[5]. Larger plastic materials not only accumulate in the environment but also gradually degrade into smaller fragments down to the nanoscale, making plastic nanomaterials more readily bioavailable at the sub-cellular level[6]. Nanoplastics have been detected in wastewaters at concentrations ranging from 0.01 to 10 μg/L. Studies have shown that nanoplastics can induce oxidative stress, leading to lipid peroxidation and DNA damage[7,8]. These effects align with the toxic properties of municipal wastewater, which can cause oxidative stress, genotoxicity, and neuroendocrine disruption[9,10]. Given these concerns, there is an urgent need for rapid and cost-effective tools to assess wastewater quality for environmental compliance, especially in the face of climate change and the emergence of new chemicals, such as plastic materials.

Peroxidases (PERs) are heme-containing enzymes involved in the elimination of the toxic reactive oxygen species H_2O_2, using electron donors (reducing agents) such as ascorbate, thiols, PAHs, and phenols[11-13]. The production of reactive oxygen species is often associated with the initiation of toxicity, and the inhibition of PER activity could serve as an early biochemical event in the manifestation of toxicity[14]. PER activity is involved in the peroxidative catalytic oxidation of H_2O_2 and requires an electron donor (A): $AH_2 + H_2O_2 \rightarrow AOH + H_2O$. The formation of AOH indicates the production of oxidized by-products, which are typically less toxic than H_2O_2. PER enzymes participate in the PER-oxidase reaction and induce oscillations in NADH (electron donor) and O_2[15]. These enzymes can oxidize a wide variety of environmental pollutants[16]. Additionally, various environmental pollutants have been shown to inhibit PER activity, leading to oxidative damage, such as lipid peroxidation and ferroptosis[17]. A previous report found that industrial effluents inhibited PER activity, leading to higher H_2O_2 levels[18]. Interestingly, the inhibition of PER by industrial effluents was linked to fish toxicity (mortality), making this enzyme a potential screening tool for large sample volumes and contributing to a reduction in fish testing for environment monitoring surveys. An interesting variation of the PER inhibition test is the restoration of PER activity following the addition of DNA. In the so-called DNA protection assay, it was revealed that 70% of industrial effluents showing DNA protection were genotoxic, as determined by a bacterial DNA repair (SOS chromotest) assay. A recent study of municipal effluents found that 90% of the tested effluent extracts inhibited PER activity, suggesting potential toxicity[19]. Of these, 60% showed DNA protection of PER activity, further suggesting the presence of genotoxic compounds in these wastewaters. This finding corroborates previous studies indicating that municipal effluents are genotoxic to fish and mussels[20,21]. These studies highlight the usefulness of this rapid assay for screening wastewater quality. In the present study, the PER assay was revisited to better understand PER inhibition by including an unsaturated lipid analog to monitor lipid peroxidation and examine the oscillations of PER intermediates. This approach will provide new information on the mechanisms of complex mixtures and the onset of oxidative damage, in addition to the detection of potential genotoxic compounds. The catalytic intermediates (compound 0, I, II, and III) oscillate over time, with compound III representing a non-catalytic intermediate that binds HOO- to the

active center, leading to O_2 and H_2O when H_2O_2 is in excess relative to the electron donor molecules[15]. Monitoring the cyclic changes in compound III, a non-catalytic intermediate, was performed to better understand the mechanisms at play during PER inhibition by municipal wastewaters.

The objective of this study was to evaluate the peroxidase-toxicity (Perotox) assay as a rapid tool for assessing wastewater quality in municipal effluents. The Perotox assay involved the addition of unsaturated lipids to investigate the effects of PER inhibition and the sustained levels of H_2O_2, as indicated by lipid peroxidation. Additionally, the protection of DNA from PER activity was examined by measuring changes in DNA strand breaks. The oscillatory changes in PER intermediate III were also assessed in an attempt to understand the mechanisms behind PER inhibition by municipal wastewater.

MATERIALS AND METHODS

Sample preparation

Horseradish PER, salmon sperm DNA, Tween-80, luminol, and bovine serum albumin were purchased from Sigma Chemicals (Ontario, Canada). Each substance was prepared at a concentration of 0.1 mg/mL in phosphate-buffered saline (PBS: 140 mM NaCl, 1 mM KH_2PO_4, and 1 mM $NaHCO_3$, pH 7.5) and stored in the dark at 4 °C for no more than one week. The DNA was dissolved in 50 mM NaCl containing 1 mM KH_2PO_4 (pH 7.4), heated at 70 °C for 15 min to ensure complete dissolution, and then stored at 4 °C. Hydrogen peroxide (H_2O_2) solutions were prepared at a 1% concentration and stored at 4 °C for no longer than one week. The PER substrate luminol stock solution was dissolved in PBS at 1 mM concentration.

Wastewater extract characteristics

Municipal wastewater samples were collected from four cities with varying population sizes as 24-h composite samples over 3 days [Table 1]. A volume of 1 L from each sample was transported to the laboratory, stored at 4 °C in the dark, and subsequently filtered using a 0.8 µm pore cellulose filter. The filtered samples were then stored in dark bottles at -20 °C until analysis. The municipal effluents were fractionated using a C18 solid-phase extraction column. A 200 mL volume was passed through a C18 cartridge containing 200 mg of sorbent, washed with 10 mL of Milli-Q water, and eluted with 1 mL absolute ethanol. The dissolved organic matter (DOC) content in the ethanol extract was quantified at 254 nm as described previously[22], using the equation: DOC (mg/L) = 0.21A + 1.25. The levels of plastic materials in the organic matter matrix (i.e., the C18 extract) were assessed using the copper fluorescence quenching method[23]. After adding 50 µM $CuSO_4$, fluorescence intensities for polypropylene (PP) (excitation: 250 nm, emission: 324 nm) and polyvinyl chloride/polystyrene (PS) (excitation: 295 nm, emission: 411 nm) were measured. Additionally, humic/fulvic acids (HA/FA) were detected at excitation/emission wavelengths of 265 and 463 nm, respectively. The fluorescence difference before and after $CuSO_4$ addition was calculated, and standard solutions of PS (20 nm diameter) and PP (100 nm diameter) were used for calibration. The results were expressed as µg PP or PS/PVC-equivalents/ mg DOC. The levels of PAHs in the ethanol extracts were determined using fixed-wavelength fluorometry[24]. Briefly, 100 µL of the ethanol extracts were placed in a dark 96-well microplate, and fluorescence was measured using a fluorescence microplate reader (Neo-2 Synergy, Biotek Instruments, USA) at the following wavelengths: excitation at 290 nm/emission at 340 nm (for light PAHs: naphthalene), excitation at 325 nm/emission at 370 nm (for medium PAHs: pyrene), and excitation at 385 nm/emission at 440 nm [for heavy PAHs: benzo(a)pyrene]. Standard solutions of PAHs were used for calibration, and the recovery rates for each PAH size class ranged between 80% and 95% during C18 extraction columns and ethanol elution.

Perotox assay

The Perotox assay was based on a previous methodology for toxicity screening of industrial effluents[18,19]. The assay involves a reaction mixture containing 0.1 µg/mL PER, albumin, and 0.0001% Tween-80 in PBS

Table 1. Physio-chemical characteristics of wastewaters

Wastewater treatment plant	Population	DOC	PS (µg/L)	$PAHs_{light}$ (µg/L)	$PAHs_{medium}$ (µg/L)	$PAHs_{high}$ (µg/L)
Lagoons	123,182	1.18 ± 0.01^a	38 ± 0.5^a	51 ± 2^a	8 ± 0.5^a	0.06 ± 0.01^a
SecA1	656,205	1.23 ± 0.02^b	16 ± 0.2^b	33 ± 13^b	7.2 ± 0.7^a	0.47 ± 0.06^b
SecA2	589,748	1.14 ± 0.02^a	11 ± 0.3^b	22 ± 2^b	3.8 ± 0.3^b	0.19 ± 0.07^c
SeCM	523,000	1.095 ± 0.012^c	10 ± 0.2^b	30 ± 3^b	3.8 ± 0.8^b	0.15 ± 0.07^c

Significant differences are indicated by different letters, while similar data share the same letter (a, b, or c). DOC: Dissolved organic carbon; PS: polystyrene; PAHs: polyaromatic hydrocarbons; SecA1: secondary aeration lagoon 1; SecA2: secondary aeration lagoon 2; SeCM: secondary membrane filtration.

(an unsaturated surfactant serving as a proxy for unsaturated lipids), combined with 0.1% H_2O_2 -0.1 mM luminol mix. DNA (0.1 µg/mL) was also added to duplicate wells to assess recovery of PER activity (DNA protection assay). The reaction media was added to the effluent extract equivalent to 10× of the C18 extract and mixed for 5 min in a clear-bottom black 96-well microplate (Synergy-4, Biotek Instruments, CA, USA). Afterward, luminol and H_2O_2 were added to initiate the reaction, which was maintained at 25 °C for 30 min. Luminescence readings and measurements at 418 nm (indicative of the PER compound III intermediate) were taken every 2 min[25]. The same procedure was repeated using ethanol extracts pre-incubated with 1 µg/mL DNA for 5 min to evaluate the influence of DNA on PER reaction rates. The DNA protection index was calculated as: PER activity with DNA/Per activity. At the end of the incubation period, 5 µL of ice-cold TCA was added to the reaction mixture and centrifuged at 10,000 × *g* for 5 min at 4 °C. The supernatant (containing DNA strands) was mixed with 10 µg/mL Hoechst dye in 100 mM KH_2PO_4 buffer (pH 8), and fluorescence was measured at 360 nm excitation/460 nm in dark microplates as described above. Standard solutions of salmon sperm DNA were used for calibration. For Tween-80 peroxidation, malonaldehyde levels were determined using thiobarbituric acid reagent methodology[26]. Briefly, 10 µL of the reaction mixture at 30 min was mixed with 90 µL of water and 50 µL of TCA 1% was added. Then, 50 µL of thiobarbituric acid (0.67%) was added, and the mixture was heated at 70 °C for 5 min. Fluorescence at 540 nm excitation and 590 nm emission was measured in dark 96-well microplates. Data were expressed as relative levels of thiobarbituric acid reactants PER well. For the PER assay, blanks contained ethanol only and $CdNO_3$ was used as a positive control (1 µg/L $CdNO_3$ decreases PER activity by 30%-40%). This Cd concentration (µg/L) aligns with the LC_{50} values (0.7-3 µg/L) for rainbow trout embryos and larvae toxicity[27].

Data analysis

The *in vitro* exposure experiments to the various influents/effluents from four cities representing different wastewater treatments were repeated three times in the absence and presence of added DNA to evaluate DNA protection. The population sizes of the cities (anonymous) and the types of wastewater treatments employed were as follows [Table 1]: aerated lagoon (Lag), two secondary activated sludge effluents (SecA), and a secondary membrane bioreactor (SecM). This study aimed to assess the impact of treatment processes in cities with different population sizes. To account for the influence of population size, an analysis of covariance (ANCOVA) was performed, with the treatment process as the main variable and population size (log-transformed) as a covariate. Critical differences between wastewater treatments from the four cities and the controls (Milli-Q water used as an operational control at the effluent containers and during C18 extraction steps) were identified using the least significant difference (LSD) test, with significance set at $\alpha \leq 0.05$. The relationships between the measured endpoints were examined using Pearson's moment correlation test, with results summarized in Table 2. All statistical analyses were conducted using the StatSoft software package (USA).

Table 2. Correlation analysis

	Population	PS	OC	$PAHs_l$	$PAHs_{med}$	$PAHs_{High}$	Perox	PeroxDNA	DNAP	TBARS	TBARSDNA	C3rate	C3DNA rate	DNAS
PS	**-0.56**	1												
OC	-0.14	0.19	1											
$PAHs_l$	-0.06	-0.23	**0.81**	1										
$PAHs_{med}$	-0.09	-0.18	**0.79**	**0.96**	1									
$PAHs_{High}$	-0.17	0.26	**0.83**	**0.68**	**0.80**	1								
Perox	**-0.34**	0.18	**0.42**	**0.5**	**0.59**	**0.59**	1							
PeroxDNA	-0.19	0.15	0.16	**0.33**	**0.41**	**0.46**	**0.38**	1						
DNAP	0.08	-0.03	-0.04	0.10	0.10	0.09	-0.29	**0.75**	1					
TBARS	**0.77**	**-0.46**	0.02	-0.02	-0.03	-0.05	**-0.52**	-0.26	0.16	1				
TBARSDNA	**0.62**	**-0.46**	**-0.31**	-0.26	**-0.32**	**-0.43**	**-0.67**	**-0.59**	-0.07	**0.65**	1			
C3rate	-0.08	**0.45**	**0.44**	0.26	0.23	**0.32**	0.30	-0.23	**-0.45**	-0.15	-0.26	1		
C3DNA rate	**0.76**	**-0.47**	0.03	-0.02	-0.04	-0.07	**-0.32**	**-0.55**	**-0.34**	**0.57**	**0.58**	0.22	1	
DNAS	**0.31**	**-0.46**	**0.46**	**0.68**	**0.50**	0.1	-0.09	-0.15	0.07	**0.31**	**0.40**	0.10	0.25	1

Significant correlations are highlighted in bold. PS: Polystyrene; OC: organic carbon; PAHs: polyaromatic hydrocarbons; DNAP: DNA protection index; TBARS: thiobarbituric acid reactants (malonaldehyde); DNAs: DNA strands.

RESULTS

Optimization of Perotox assay

The Perotox assay includes the addition of an unsaturated detergent (Tween-80) as a proxy for unsaturated lipids to evaluate lipid peroxidation [Figure 1]. This assay assesses the effects of PER inhibition and sustained H_2O_2 levels on protein, PER intermediate analysis (418 nm), and DNA protection. The consequences of sustained H_2O_2 levels due to PER inhibition were measured by lipid peroxidation and DNA strand break formation. Moreover, the assay enables real-time monitoring of PER states by tracking absorbance changes at 403 and 418 nm, corresponding to the native and component III (CIII) intermediate states of PER, respectively. CIII is a non-catalytic intermediate involved in the (spontaneous) transformation of $2H_2O_2 \rightarrow O_2$ and $2H_2O$ when the reducing substrate is deleted relative to H_2O_2. This new Perotox "version 2.0" biosensor offers a more detailed assessment of PER activity under oxidative stress at the protein/enzyme, DNA, and lipid levels.

Functional characteristics of the Perotox assay

Following the addition of H_2O_2 and luminol, a rapid increase in luminescence is observed, followed by a decrease over time [Figure 2A]. During the reaction,

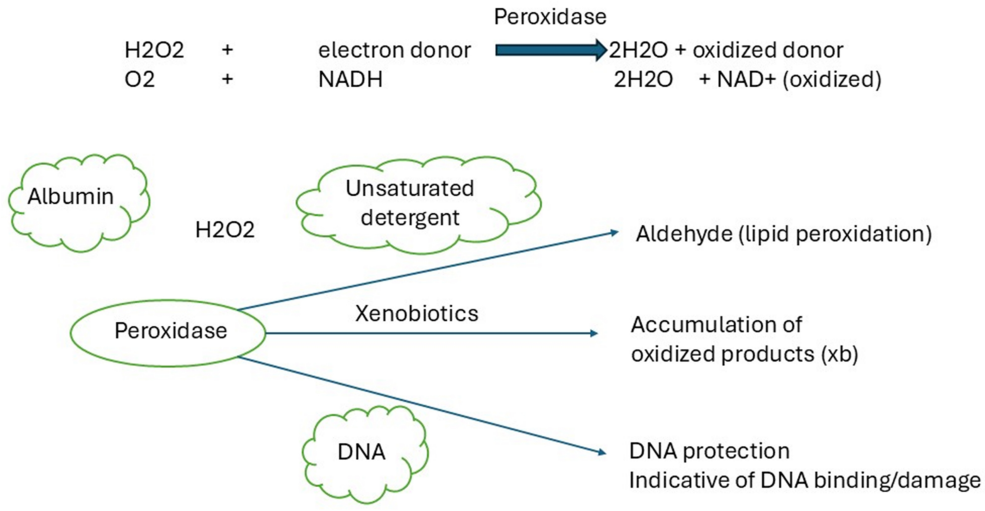

Figure 1. Principle of the Perotox assay. The Perotox enzyme assay is based on the production of oxygen radicals during the PER reaction of H_2O_2 and a reducing substrate. Both H_2O_2 and the oxidized products could, in turn, oxidize various proxy substrates in addition to luminol, such as an unsaturated lipid (Tween-80), protein (albumin), and DNA. The oxidation of unsaturated carbons of Tween-80 detergent leads to peroxidation (aldehyde formation), while interaction with DNA leads to DNA strand breaks. The addition of DNA during the PER reaction with H_2O_2 and lumino/fluorogenic substrate (luminol or dihydrofluorescein) could also protect PER against inhibition by the xenobiotics, the so-called DNA protection assay. Perotox: Peroxidase-toxicity; PER: peroxidase.

increasing the amount of unsaturated detergent Tween-80 results in the formation of malonaldehyde (hydrolysis of peroxides) [Figure 2B]. This rise in malonaldehyde levels was also accompanied by an increased formation rate of PER compound III [Figure 2C], suggesting that not all H_2O_2 is oxidized by PER and luminol. Instead, PER compound III followed a secondary pathway, leading to the degradation of H_2O_2 to H_2O and O_2 when the luminol concentration is lower than that of H_2O_2. In the presence of DNA, luminescence decreases over time, similar to the pattern observed with PER alone [Figure 2D]. The levels of DNA strand breaks, as determined by the TCA-precipitation method, also increased as the DNA concentration rose [Figure 2E]. As with PER alone, the formation of compound III over time increased with the addition of DNA, further suggesting that H_2O_2 was somewhat in excess relative to luminol when DNA or Tween-20 was present [Figure 2F].

Intermediates of PER activity

The native form of the PER enzyme and its intermediate compound III (CIII) were evaluated during the PER reaction in the presence of DNA and municipal effluent extracts [Figure 3]. During this reaction, the PER enzyme transitioned through various intermediates, from its native form (characterized by an absorbance at 403 nm) to various intermediate compounds such as C0, CI, CII and CIII (characterized by an absorbance at 418 nm for CIII intermediate). The native form acts as a link between the peroxidation (C0, CI, CII) and oxidation (CIII) reactions: CIII ⇔ Native → CO → CI → CII ⇔ CIII. Under normal conditions, PER accelerates the oxidation of luminol with H_2O_2 over time. Intermediate CIII oscillates with a period of 200 s (3.3 min) under these conditions, i.e., in the presence of PER (with albumin), its substrates (H_2O_2 and luminol), but without added Tween-80 or DNA. Notably, the levels of CIII did not change over time. The same inverse pattern for the native form of PER was also observed (results not shown). Upon the addition of DNA, an increase in CIII was observed over time (indicating a faster formation rate of CIII). Oscillations were still observed but with lower amplitudes and a longer period of 250 s (4.2 min). When municipal effluent extract was added to the PER reaction mixture, a similar increase in the rate of CIII formation was observed. The oscillations remained comparable in amplitude and periodicity to those observed in the PER reaction without additives. In the presence of both DNA and municipal effluent

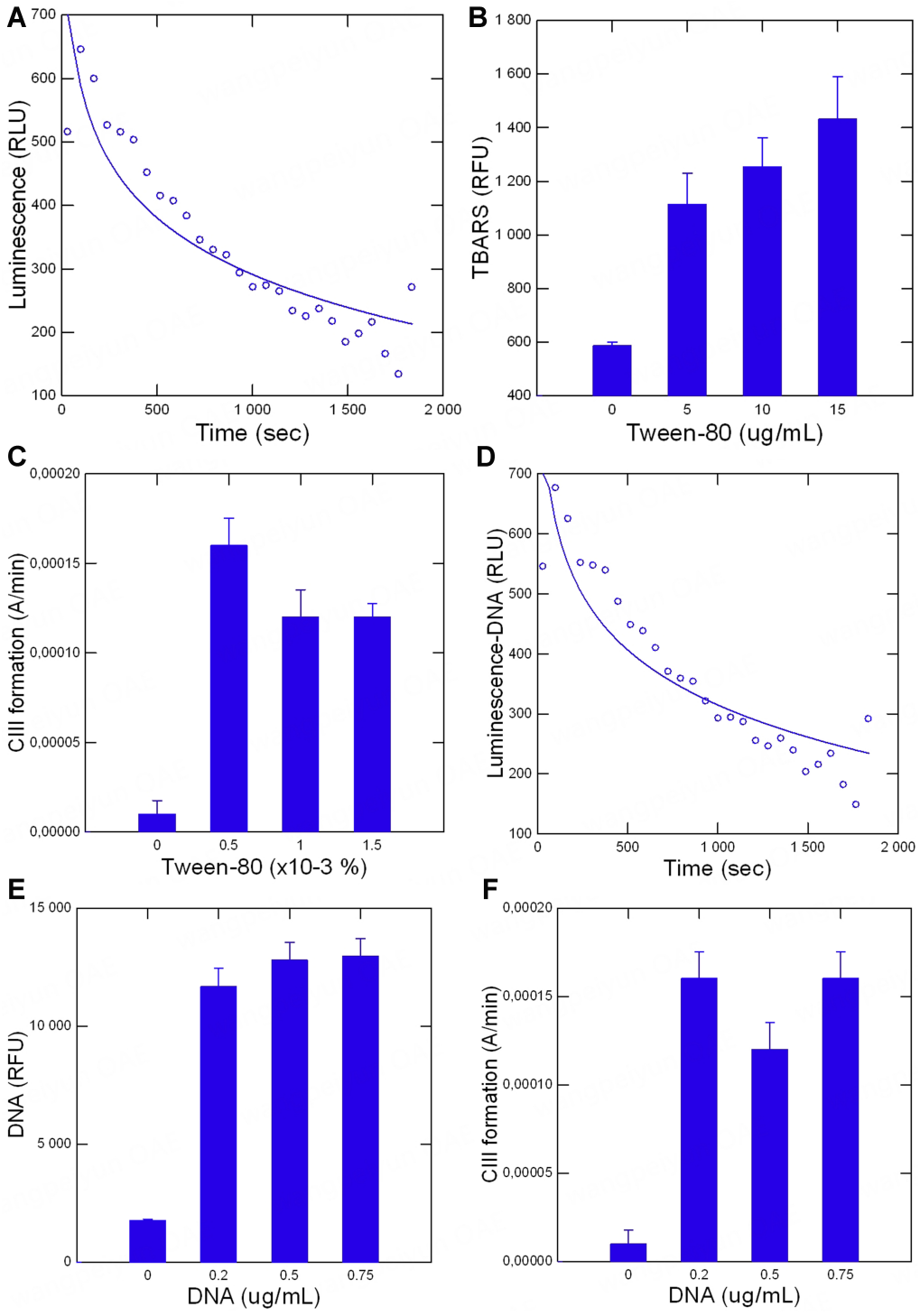

Figure 2. PER reaction characteristics in the presence of Tween-80 and DNA. Perotox assay in control (absence of toxicants) conditions. Change in luminescence (A), production of malonaldehyde (B), and formation of Complex III (C) in the absence of exogenous DNA. Change in luminescence (D), acid-soluble DNA (E), and formation of Complex III (F) in the presence of exogenous DNA. PER: Peroxidase; Perotox: peroxidase-toxicity.

extracts, the rate of CIII formation was also observed, with oscillations displaying larger amplitudes compared to the PER-DNA reaction but retaining a similar periodicity of 4.2 min. These observations

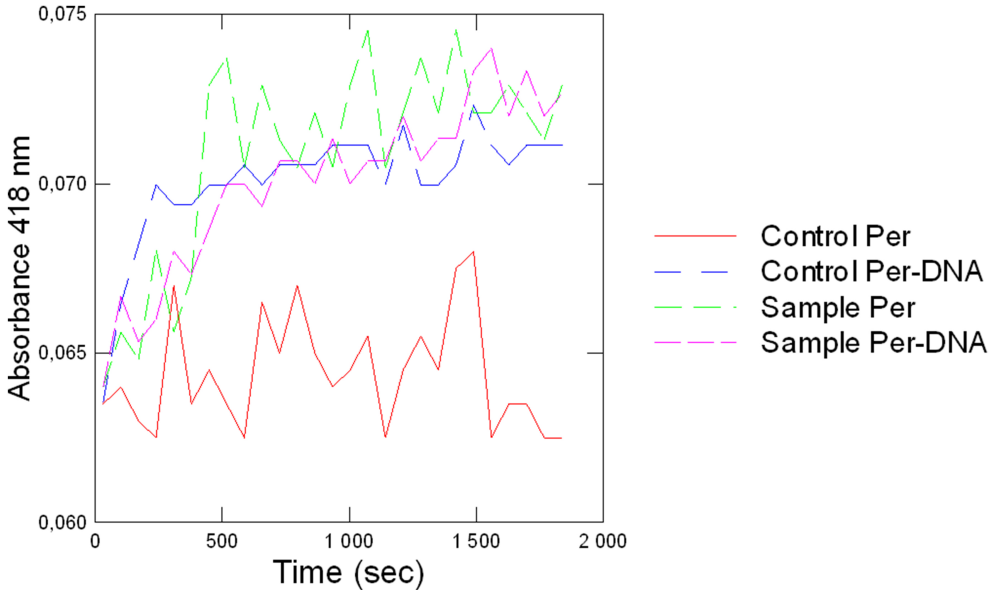

Figure 3. Oscillation of compound III over time. The change in compound III (418 nm) intermediate was monitored over time with PER alone, PER with DNA in the controls, and in the presence of municipal effluent samples. PER: Peroxidase.

suggest that the presence of DNA either interacts with luminol, reducing its availability to PER and thereby increasing the rate of CIII formation, or induces molecular crowding effects due to DNA strand interactions. Fractal frequency analysis revealed fractal dimensions (FDs) of 1.344, 1.215, 1.433, and 1.174 for PER, PER-DNA, PER-Effluent, and PER-DNA-effluent samples, respectively[28,29]. The reduction in FD was most pronounced for the PER-DNA-effluent (0.259) compared to PER-DNA (0.129), indicating that DNA interactions with the effluents and luminol substrates lead to steric effects, which limit substrate availability over time.

Application of the Perotox assay on municipal wastewaters

The Perotox assay was tested on municipal wastewaters subjected to different treatments [Table 1]: lagoons (Lag), Secondary aerated lagoons (SecA1 and A2), and secondary membrane filtration (SecM). All these effluents underwent primary treatment involving particle sedimentation. The wastewater effluents were characterized by measuring dissolved organic carbon (DOC), PS materials, and light, medium, and heavy PAHs [Table 1]. The wastewater originated from Canadian cities with different population sizes, all of which utilized aeration lagoons followed by secondary aeration or membrane filtration. A strong correlation was observed between population size and effluent flow rates ($r = 0.94$; $P < 0.001$). DOC levels were higher in the Lag and SecA1 treatments compared to SecA2 and SecM treatments. However, DOC levels showed no significant relationship with population size. The concentrations of PS materials in the organic matrix were also evaluated. PS levels were significantly higher in the Lag treatment compared to the other lagoons employing a secondary treatment. Moreover, PS levels were negatively correlated with population size ($r = -0.83$), suggesting that smaller cities achieve less effective removal of PS materials. The same pattern was observed for light and medium PAHs, with weak positive correlations with population size ($r = 0.39$ for light PAHs and $r = 0.42$ for medium PAHs). For heavy Pahs, the Lag treatment exhibited lower levels compared to other sites, while the SecA1 treatment revealed the highest PAH levels. No significant trend was found between heavy PAH levels and population size.

The impact of municipal effluents (at 10× concentration) from different wastewater treatment types (Lag, SecA1, SecA2, and SecM) was examined using the Perotox assay [Figure 4]. PER activity was significantly reduced by all wastewater treatment types, with SecA2 causing the most severe inhibition [Figure 4A]. PER activity was negatively correlated with the population size (r = -0.9), suggesting that water quality impacts were stronger with larger populations. All treatment types significantly increased the peroxidation of the unsaturated detergent, with SecA2 treatment resulting in the greatest damage [Figure 4B]. The levels of detergent peroxidation were significantly correlated with population size (r = 0.57). The formation rate of CIII was significantly reduced only with SecM treatment, with no effect observed from population size.

Influence of DNA addition to the Perotox assay

A small decrease in PER activity was also observed in the presence of exogenous DNA, though the decrease was less pronounced compared to PER alone [Figure 5A]. The levels of thiobarbituric acid reactants were also elevated across the various treatment processes, with SecA2 showing the highest levels of detergent (lipid) peroxidation [Figure 5B]. The formation rate of the CIII intermediate (which occurs when the co-substrate luminol decreases relative to H_2O_2) was elevated in the SecA2 treatment, indicating that excess H_2O_2 (resulting in lower PER activity) was maintained for longer periods [Figure 5C]. The DNA-PER activity was correlated with the DNA protection index (r = 0.75), detergent peroxidation (r = -0.59), and the CIII formation rate (r = -0.55). The DNA protection index was generally higher than in controls, with SecA1 and SecM effluents showing significantly higher levels [Figure 5D]. The levels of acid-soluble DNA were significantly higher in the SecA1 and SecA2 effluents [Figure 5E]. Although the DNA protection index was elevated by the various effluents, it was not significantly correlated with DNA strand formation, suggesting that the PER protection mechanisms did not always lead to DNA strand breaks [Figure 5E]. Instead, DNA strand formation was significantly correlated with PER-DNA activity (r = -0.36), peroxidation (r = 0.4), and population size (r = 0.43), indicating that reactive oxygen species (H_2O_2), oxidative damage (LPO), and effluent origin (population size) were associated with DNA strand break formation.

DISCUSSION

Mode of action of PER inhibition

The Perotox assay is a simple biosensor designed to evaluate water quality in complex mixtures, such as effluents. The principle behind the assay is based on the observation that PER inhibition, which reduces the rate of H_2O_2 elimination, leads to increased oxidative damage, such as lipid peroxidation (Tween-80) and DNA breaks. In a previous study[18], the Perotox assay successfully identified toxic effluents in rainbow trout when PER activity was reduced, highlighting its potential as an alternative method to reduce the need for fish in toxicity testing. Furthermore, inhibition of PER reaction by primary- and secondary-treated municipal wastewaters (Singapore) has also been observed, further supporting its applicability for screening municipal wastewaters[30]. In the absence of wastewater extracts, PER intermediates oscillate between compounds 0, I, II, and III, eventually returning to compound 0, as shown in Figure 2. CIII formation occurs when the reducing substrate concentration is lower than that of H_2O_2. leading to non-catalytic formation of O_2 and H_2O, similar to catalase activity[15]. However, unlike catalase, this step is non-catalytic and does not substantially enhance the production rates of O_2 and H_2O. In the presence of DNA, which can interact with luminol (3-aminophthalate) via H bonds at the amino group, the formation rate of CIII was inversely related to DNA-Per activity (r = -0.55), supporting the view that CIII formation was involved with PER activity inhibition. When DNA was present, CIII levels increased, and the cyclic changes in CIII were maintained, though with lower amplitudes. The FD of the PER reaction decreased, as expected, with the addition of DNA (FD = 1.215) compared to PER reaction alone (FD = 1.344), suggesting that the increased complexity of the reaction environment leads to limited substrate availability for PER. The addition of effluent samples and DNA further decreased the FD down to FD = 1.174. PER activity was somewhat lowered in the presence of DNA, indicating that luminol and H_2O_2 reacted less efficiently. This finding

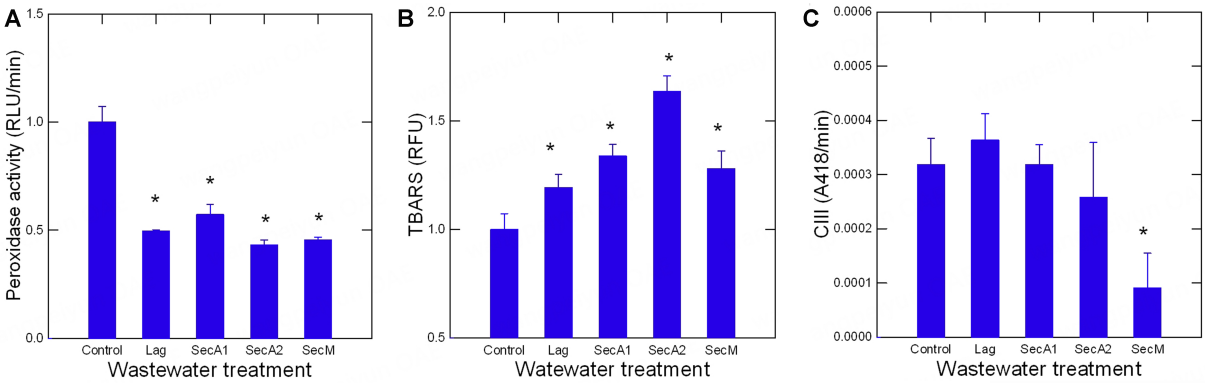

Figure 4. Influence of municipal wastewater extracts on the PER assay. The Perotox assay was used to determine the potential toxic properties of municipal influents and effluents (A). The formation rate of CIII (B; 418 nm) and the levels of aldehydes (C; TBARS) were determined. The control represents the PER assay in the presence of the vehicle (ethanol). The data are expressed as the mean with the standard error. The star symbol * indicates significance relative to controls. PER: Peroxidase; Perotox: peroxidase-toxicity; TBARS: thiobarbituric acid reactants (malonaldehyde).

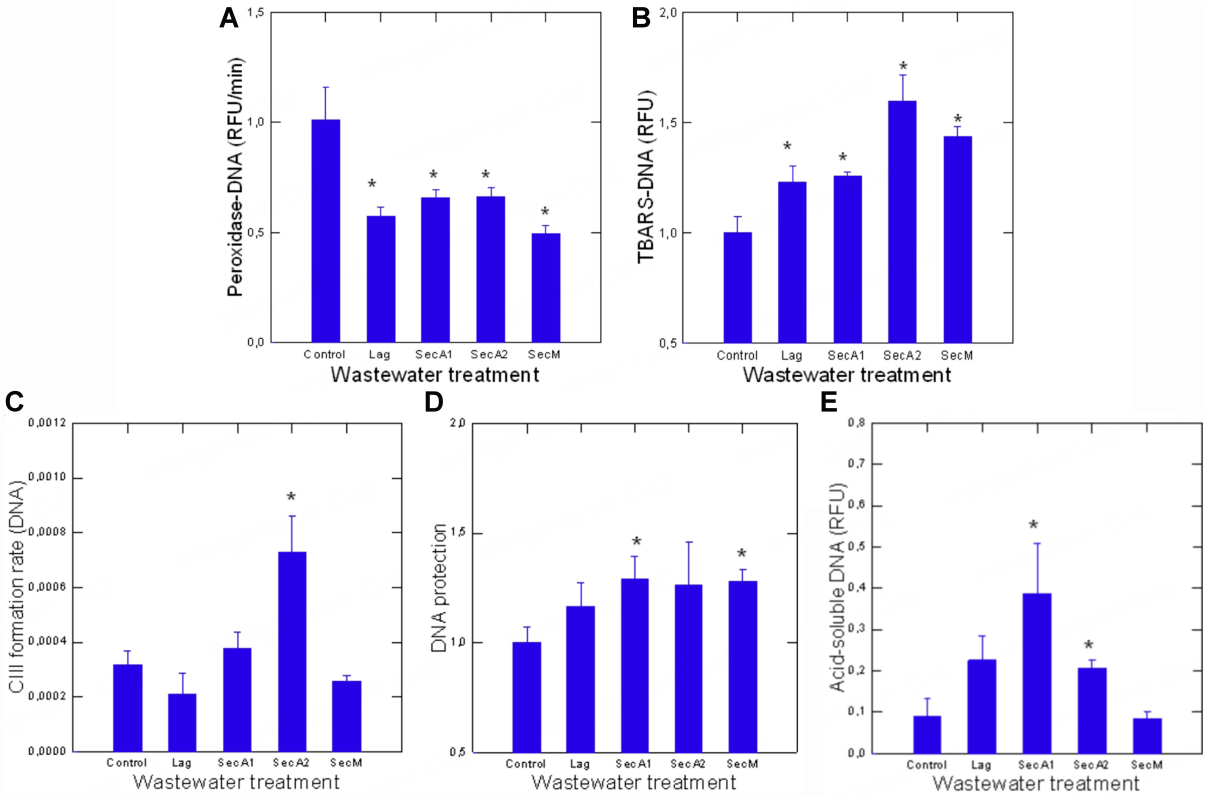

Figure 5. Influence of municipal wastewaters on the peroxidase toxicity in the presence of DNA. The Perotox assay was tested on municipal influents and effluents in the presence of DNA (A) with detergent peroxidation (B). The formation rate of CIII (C; 418 nm), the DNA protection index (D), and the formation of acid-soluble DNA (E) were determined. The control represents the PER assay in the presence of the vehicle (ethanol). The data are expressed as the mean with the standard error. The star symbol * indicates significance relative to the controls. Perotox: Peroxidase-toxicity; PER: peroxidase.

aligns with previous observations that DNA strands reduce the chemoluminescence reaction rate of luminol with H_2O_2[31].

The influence of municipal wastewaters on PER

Following the addition of municipal effluents with or without DNA, CIII oscillations are maintained at lower amplitudes, with a steady increase in CIII formation over time. This suggests that PER activity can be inhibited by various chemicals in effluents, potentially due to a reduction in the spatial domain of the enzyme (FD, especially in the presence of complex macromolecules such as DNA) and the formation of non-catalytic CIII over time. Ferroptosis, a form of programmed cell death, occurs when Fe, H_2O_2, and decreased PER activity lead to the accumulation of lipid hydroperoxides[32]. This mechanism could represent a fundamental basis for cell death induced by Fe release (from hemoproteins such as PERs) and the formation of lipid hydroperoxides due to persistent H_2O_2 in cells. Heme proteins have been shown to bind various metals, releasing Fe during heme breakdown (catabolism) via the heme oxygenase pathway[33]. Several chemicals, including herbicides (e.g., paraquat), detergents (e.g., Brij-96 and Tween-20), phenol, and heavy metals such as Hg, Co, and Ni, have been reported to inhibit PER activity[13]. Conversely, some pro-oxidant compounds such as Cd, Mg, and Zn have been observed to increase PER activity, which could deplete cellular antioxidants over the long term. In algae, PER activity was commensurate with copper and lead levels in stormwater, suggesting that certain chemicals can induce PER activity[11]. While reduced PER activity in control samples was associated with lower biomass, increases in PER activity above the normal range were also linked to reduced biomass (algal growth). This suggests that deviations in PER activity from the normal range may be harmful due to the accumulation of either H_2O_2 or oxidized substrates.

Wastewater contaminants and PER activity

This study found an inverse relationship between PER activity and population size (and corresponding flow rates), with plastic materials being more common in smaller cities. Smaller wastewater treatment plants are perhaps ill-equipped to reduce plastic pollutant loads in effluents[34]. The presence of plastics was linked to increased CIII formation rates in the absence of DNA ($r = 0.45$), indicating that plastics could also reduce PER activity by enhancing CIII formation rates. Exposure of Vicia faba L seedlings to microplastics led to reduced PER activity and root sugar content[35]. Moreover, the addition of Cd with microplastics also resulted in decreased PER activity. In mouse primary hepatocyte cells, 20 nm nanoplastics demonstrated a pronounced ability to induce lipid peroxidation, due to the reduced activity of antioxidant enzymes such as superoxide dismutase, catalase, and perhaps glutathione PER[36]. However, in this study, PER activity showed a negative correlation with thiobarbituric acid levels, both with and without added DNA, suggesting that decreased enzyme activity (and consequently reduced removal of H_2O_2) leads to the oxidation of unsaturated Tween-80 in the reaction mixture. In wastewater samples, there was no direct indication that plastics inhibited PER activity, as no correlation was observed. However, recent findings suggest that PER enzyme can adsorb onto the surfaces of amino-coated (i.e., positively charged) PS nanoparticles[37]. This adsorption forms a protein corona on the plastic nanoparticles, reducing their Zeta potential, surface roughness, and PER activity. These results imply that plastic nanoparticles could induce oxidative stress and cellular damage by inhibiting PER activity. Further research is needed to elucidate the role of plastic size and shape in modulating PER activity. Interestingly, the negative correlation between PS plastics and lipid hydroperoxide formation (in the absence or presence of DNA) suggests that PS plastics could in some way adsorb other chemicals, thereby preventing inhibition of PER activity. Plastics are well known to act as chemical vectors, binding various substances on their hydrophobic surfaces[38]. For example, plastics can adsorb chemicals with logKow values between 3.3 and 9, such as perfluoroalkyl substances (PAFSs), hexachlorocyclohexanes (HCHs), and polycyclic aromatic hydrocarbons (PAHs). During weathering, the formation of C-O and N-H groups on plastic surfaces enhances their capacity to adsorb metals such as Cr, Pb, and Co. It is, therefore, possible that plastic materials in effluents could act as chemical sinks and as vectors for pollutant transport. Notably, municipal effluents often contain unsaturated surfactants and detergents, which can also contribute to peroxidation[39]. This hypothesis could be tested by directly applying the Perotox assay to wastewater samples, without the addition of Tween-80, to measure lipid

peroxidation levels.

CONCLUSIONS

The Perotox assay is proposed as a rapid screening tool for complex liquid mixtures, such as effluents and leachates. An unsaturated detergent was included in the reaction mix to evaluate the impact of PER inhibition (indicated by lower H_2O_2 removal rates), which serves as a proxy for the formation of lipid hydroperoxides from polyunsaturated lipids. The assay revealed that inhibition of PER activity is associated with decreased amplitudes in CIII oscillations, an increased rate of non-catalytic intermediate CIII formation, reduced spatial dimensions, and the formation of lipid peroxidation products. These findings form the toxicological basis for understanding the effects of PER inhibition in organisms exposed to various environmental pollutants. The addition of exogenous DNA during the reaction did not result in significant changes to PER activity, suggesting it could be used to identify chemical interactions that prevent PER inhibition. This simple biosensor represents a convenient and cost-effective tool for screening various complex mixtures and emerging contaminants that compromise water quality and toxicity.

DECLARATIONS

Authors' contributions

Extraction of municipal wasterwaters for the Perotox assay, data analysis, and revision of the draft manuscript: André, C.

Collection and preparation of municipal wastewatetrs, supervision, funding acquisition, and revision of the draft manuscript: Smyth, S.A.

Funding acquisition, data acquisition, data analysis and preparation of the draft and final manuscript: Gagné, F.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of interest

Gagné, F. is an Editorial Board Member of the journal *Water Emerging Contaminants & Nanoplastics*. Gagné, F. was not involved in any steps of editorial processing, notably including reviewer selection, manuscript handling, and decision making. The other authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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