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The role of lysosomal membrane stability, malondialdehyde levels and DNA damage as pollution biomarkers in evaluating biological cleaning products using *Mytilus galloprovincialis*

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ABSTRACT

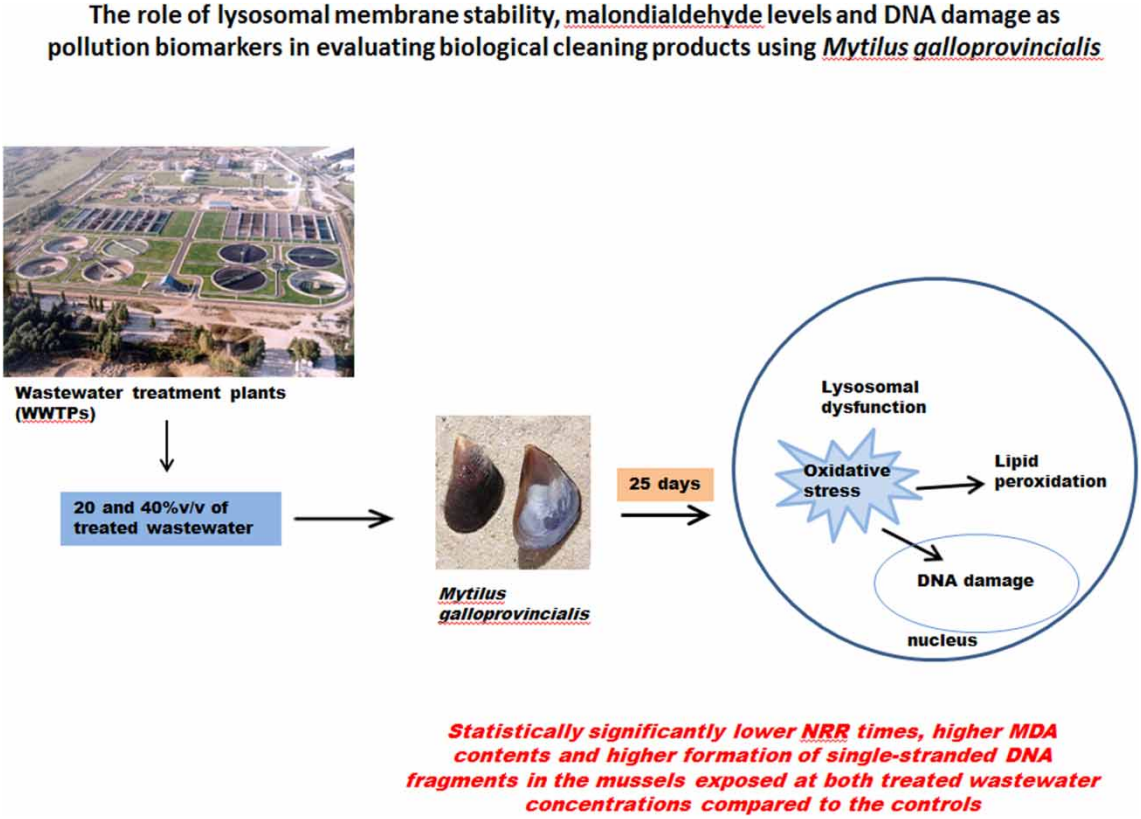
The possible effects of wastewater treatment products on mussels (*Mytilus galloprovincialis*) were studied. Three common pollution biomarkers were used: neutral red retention assay, malondialdehyde contents and DNA damage through comet assay. Two groups of mussels were treated in the laboratory for 25 days with 20 and 40 %v/v of treated wastewater collected after chlorination, and the control mussels formed a third group. The results showed statistically significantly lower neutral red retention times, higher malondialdehyde contents and higher formation of single-stranded DNA fragments in the mussels exposed at both treated wastewater concentrations compared to the controls.

Key words: biological cleaning, comet assay, MDA, NRR

HIGHLIGHTS

- Statistically significantly lower NRR times were found in exposed mussels compared to controls.
- Statistically significantly higher MDA values were found in exposed mussels compared to controls.
- Remarkable sensitivity of the haemocytes to the formation of single-stranded DNA fragments were observed in the mussels exposed at both biological sample purification samples compared to the control ones.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Wastewater treatment plants (WWTPs) can release complex chemical mixtures into receiving waters, including heavy metals such as Cu, Zn, Ni and Cr, and end-of-life industrial products (Karvelas *et al.* 2003). WWTP effluents (WWTPEs) can also include polycyclic aromatic hydrocarbons (PAHs), pesticides, surfactants, steroids and pharmaceuticals (Abessa *et al.* 2005; Metcalfe *et al.* 2010; Lara-Martín *et al.* 2014; Zaborska *et al.* 2019; Aguirre-Martinez & Martín-Díaz 2020), or even endocrine disruptors and hospital waste products (Kümmerer 2001), many of which have also been found in receiving environments (Kolpin *et al.* 2002). Among organic pollutants, pharmaceutical and personal care products (PPCPs) (Alayu & Yirgu 2018) cannot be easily degraded by existing treatments (Ebele *et al.* 2017). WWTPEs include sand, suspended solid particles, natural organics that are consumed by microorganisms causing a parallel reduction of dissolved oxygen in the water body, pathogens responsible for transmitting diseases to humans and other organisms, and eutrophication-causing nutrients – e.g., phosphorus and nitrogen (Cooke 2006; Gillis 2012). Such compounds are characterized by pronounced persistence against chemical/biological degradation, high environmental mobility, strong tendencies for bioaccumulation in human and animal tissues, and significant impacts on human health and the environment even at extremely low concentrations (Katsoyiannis & Samara 2004, 2007).

WWTPs are one of the most effective ways of dealing with water resource pollution, and are designed to remove pollutants from municipal wastewater and release a clean effluent. Wastewater treatment has three main stages: primary treatment is intended to remove bulky solids, sand, etc. In the secondary or biological treatment stage the organic components of biological oxidation are removed. In tertiary treatment nutrients – e.g., phosphate, nitrate, borate and silicate – are removed.

Due to the cost of biological treatment, many WWTPs, including that at Thessaloniki, Greece, stop after the secondary stage and apply final disinfection with chlorine. The basic parameters usually assessed in the effluent are (a) COD – the amount of oxygen required to oxidize the water's chemical content – and (b) BOD – the amount of oxygen used by microorganisms to oxidize the water's organic load. Most previous studies of

WWTPEs refer, among other things, to estimates of these two parameters, but few studies exist on the evaluation of WWTPE effects on biological systems.

WWTP influents and effluents include detectable concentrations of persistent pollutants and metabolites – both known and undiscovered – which may affect ecosystem health (Narayanan *et al.* 2022). Despite the fact that WWTPEs only contain the pollutants mentioned at trace concentrations, they seem to be toxic to a variety of organisms. Onchoke *et al.* (2022) supported the need to design analytical methods to detect pollutants. The use of biomonitoring assays may also enhance the characterization of such discharges. Organisms used as bio-indicators include mussels, which are characterized by the ability to accumulate various pollutants (Reichwaldt & Ghadouani 2016) due to their capacity of water filtering (Beyer *et al.* 2017).

The biomarker neutral red retention assay (NRR) has been applied to evaluate heavy metal or organic effects in both field (Koagouw *et al.* 2021) and laboratory studies (Parisi *et al.* 2021). The dye retention time in lysosomes is influenced mainly by the presence of pollutants.

Malondialdehyde (MDA) is a very common lipid peroxidation product that provokes damage to DNA or cellular proteins, and is considered a secondary carbonyl degradation product of lipid peroxidation of cell membranes (Alexandrova & Bochev 2005). Because of this, MDA lipid peroxidation products have been proposed to reflect the oxidative status of exposed species (Cossu *et al.* 2000) and may be regarded as the most commonly used biomarker for measuring oxidative stress levels (Almeida *et al.* 2005; Khalil 2015).

An important result of oxidative stress in mussels is DNA damage (Frenzilli *et al.* 2001) in relation to water quality in the environment where they live. If DNA damage is not repaired, a cascade of biological effects can be induced at cell or organism level, and, finally, population level. Comet assay – single cell gel electrophoresis assay – is an established biomarker in pollution biomonitoring studies (Silva dos Santos *et al.* 2022).

The goal of this study was to evaluate the effects of WWTPEs using established cellular, biochemical and genotoxic pollution biomarkers on living organisms.

2. MATERIALS AND METHODS

2.1. Thessaloniki WWTP

Thessaloniki's WWTP is in Sindos, near the French/Ehedoros river, and is supervised by the Thessaloniki Water Supply and Sewerage Company. It serves about 1 million people, treating 120,000–150,000 m^3/d of raw sewage. About 5–10% of the influent comes from industry. The plant also receives the greatest part of local urban runoff, composed mainly of atmospheric deposition and traffic-related emissions from the road surface. Treatment includes screening, grit removal, primary sedimentation without chemical coagulants, conventional activated sludge treatment and effluent disinfection with chlorine (gas). The treated wastewater is discharged to the Thermaic Gulf via a channel. Sewage sludge (primary plus excess activated) is digested anaerobically, thickened and dewatered (Karvelas *et al.* 2003). Most of the sludge is deposited in a municipal landfill, while its use as soil amendment is also under consideration by the local authorities (Katsoyiannis & Samara 2007).

2.2. Mussel collection and experimental procedure

Mussels – *Mytilus galloprovincialis* (5–6 cm long) – were collected from Chalastra (west of Thessaloniki), and transported to the laboratory within 1 hour of collection. They were maintained without food for 1 week, to become acclimated to laboratory conditions.

After acclimation the mussels were divided into 3 groups (45 mussels/group) and placed in static tanks containing 10 L of aerated seawater. The control group (A) consisted of unexposed mussels. Mussel group B was kept for 25 days in 20% v/v and group C in 40% v/v of treated effluent collected after chlorination at the WWTP. The water was replaced every 2 days, and fresh quantities of WWTPE and food diluted/dissolved in seawater were added. Preliminary experiments using similar effluent dilutions were conducted and the results showed significant changes in the values of the biomarkers applied. A mortality test (LC50 determined by Probit analysis) was performed in order to estimate the range of WWTPE concentration that produced no mortality. 5 groups of mussels (10 mussels/group) were placed in static tanks containing 10 L of aerated seawater and exposed for 18 days to different concentrations of WWTPE (20, 40, 60, 80 and 100% v/v), while a control group was without WWTPE. The water was changed every 2 days, and fresh solutions of WWTPE and food added. Mussels were considered dead when they did not respond to the squeezing of the valves, after the valves have gaped, and were then removed from the tanks. The results showed that mortality observed in mussels exposed to low WWTPE concentrations (20 and 40% v/v) was negligible, compared to mussels exposed to higher concentrations.

Concentrations between 20 and 40% v/v could, thus, be considered non-lethal and were, therefore, chosen to investigate the pre-pathological effects of WWTPE on mussel hemocytes by applying pollution biomarkers, to exclude cell death as a parameter that could interfere with the results.

2.3. NRR assay

The NRR assay was performed following Lowe & Pipe (1994), with small modifications. Hemolymph was withdrawn from the posterior adductor muscle of 10 mussels in physiological saline, to obtain a 50/50 solution of cell/physiological saline suspension. The NRR time was measured individually for these mussels and the mean taken as the NRR time for the whole group.

2.4. MDA determination

Hemolymph was collected from the posterior adductor muscle of each mussel in a group, using a sterilized syringe, and placed in plastic tubes. 1 mL of hemolymph was then centrifuged for 10 min at 900 g and the supernatant collected.

MDA was detected in the supernatant using the method described by Niehaus & Samuelsson (1998). Hemolymph from 3 mussels was pooled and centrifuged at 900 g for 10 min for each measurement. 1 mL of the supernatant was mixed with 2 mL of trichloroacetic acid (TCA) – thiobarbituric acid (TBA)–HCl (15% w/v TCA, 0.375% w/v TBA in 0.25 N HCl), followed by incubation at 100 °C for 15 minutes. The samples were then cooled at room temperature and measured spectrophotometrically at 535 nm. The results were expressed as μmol-MDA/mL hemolymph.

2.5. Comet assay

The method described by Singh *et al.* (1988) was followed with some modifications. The presence of comets was determined in hemocyte suspension using a fluorescent microscope. Four slides were analyzed for each group. All slides were coded and the whole slides were scanned randomly. At least 250 cells per slide were analyzed. The comets on each slide were scored visually as belonging to one of five predefined classes according to tail intensity, with values from o (undamaged) to 4 (maximally damaged). The proportion of DNA in each tail was estimated using Tritek CometscoreTM 1.5 (TriTek Corporation, USA).

2.6 Data analysis

Duncan's test ($p<0.05$), breakdown and one-way ANOVA were used to test NRR assay data. For the statistical analysis of MDA contents data, post-hoc multiple comparison (Bonferroni, $p<0.05$) testing was used where significant differences were detected in the ANOVA (GraphPad Instat 2.0). The Tukey test (one-way ANOVA, $p<0.01$) was used to compare the grade of DNA damage between control and exposed cells. Simple linear correlation (Pearson's test) based on mean values was used to establish significant, biological response relationships. Analysis was done using StatSoft Inc. (2004) STATISTICA (Data Analysis Software System), Version 7.

3. RESULTS

3.1. NRR assay

The NRR time for each group was the time after applying the NR probe, when dye was lost from the lysosomes to the cytosol, in at least 50% of the cells examined. Lower NRR times were found in hemocytes of mussels treated with both dilution levels of WWTPE (ANOVA, Duncan's test, $p<0.05$) (Figure 1).

3.2. MDA contents

Statistically higher MDA contents were found in mussels treated with both dilution levels of WWTFE than in the controls (Bonferroni, $p<0.05$) (Figure 2).

3.3. Comet assay

Tukey's test ($p<0.01$) indicated marked hemocyte susceptibility to DNA damage caused by treatment with both dilution levels of WWTPE compared to the controls (Figure 3).

3.4. Correlation analysis

The correlation coefficient analysis between the applied oxidative stress parameters (Pearson's test, $p<0,05$) showed:

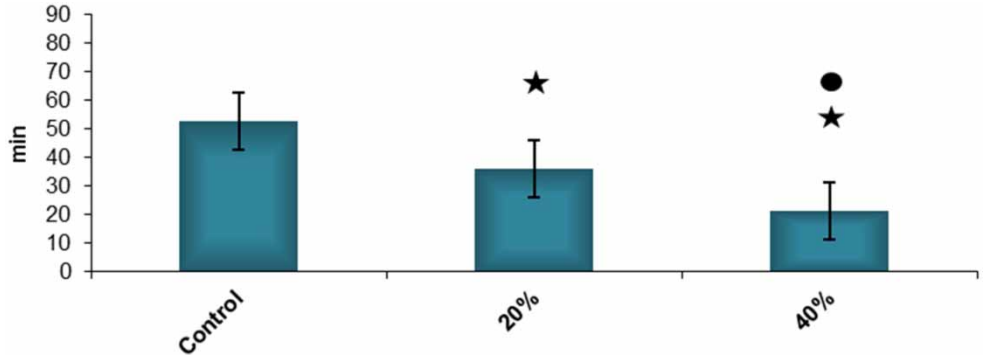


Figure 1 | NRR values (min) of the hemocytes of mussels (*M. galloprovincialis*) exposed to 20 and 40% concentrations of WWPTE. ★ indicates significant difference between the control value and that observed after exposure (Duncan's test, $p<0.01$).

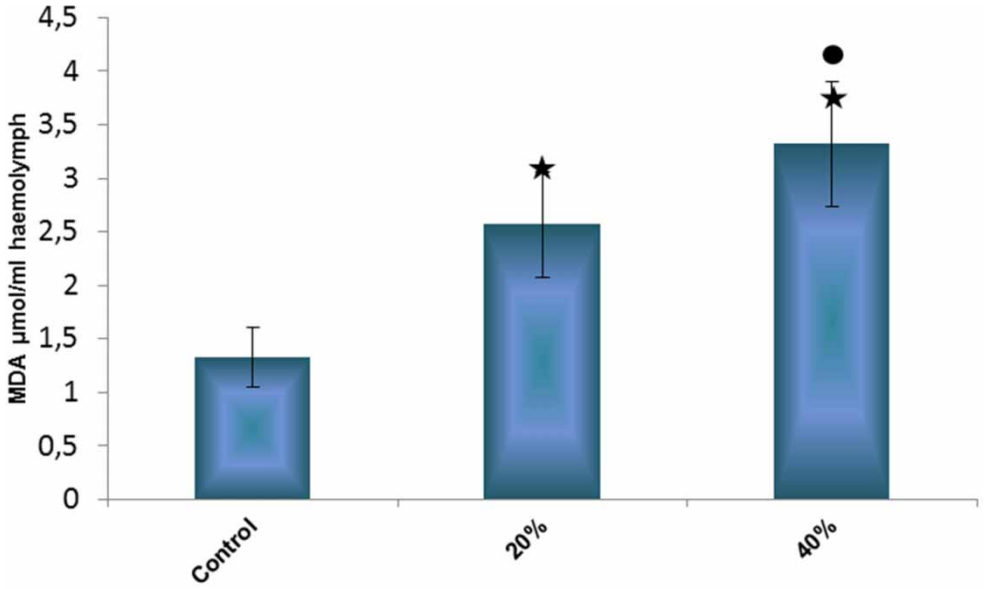


Figure 2 | MDA content in hemolymph of mussels (*M. galloprovincialis*) exposed to 20 and 40% concentrations of WWTPE. ★ indicates significant difference between the control value and that observed after exposure (Bonferroni, $p<0.05$); ● indicates significant difference between mussels exposed to different WWTPE concentrations (Bonferroni, $p<0.05$).

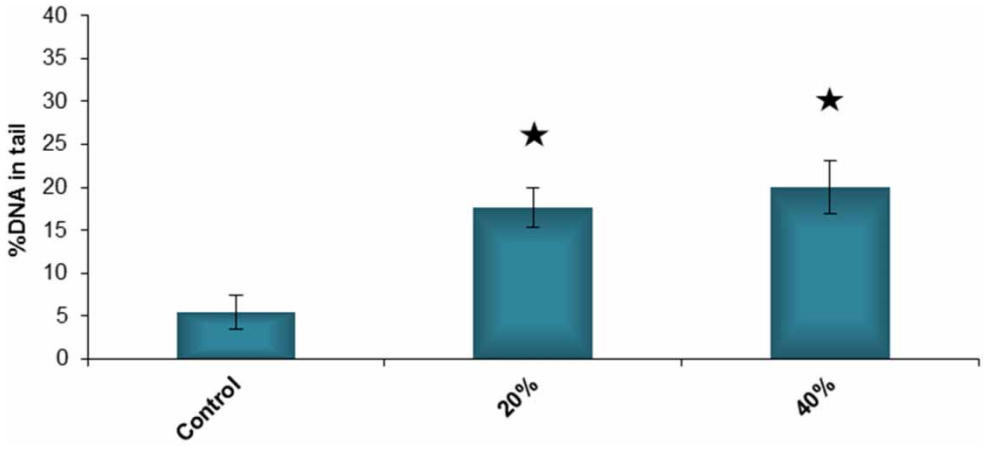


Figure 3 | DNA damage of isolated hemocytes of *M. galloprovincialis* exposed to 20 and 40% concentrations of WWPTE. ★ indicates significant difference between the control value and that observed after exposure.

1. A strong negative correlation between NRR times and MDA contents (r=−1,00).
2. A strong negative correlation between NRR times and DNA damage levels (r=−1,00).
3. A strong positive correlation between DNA damage levels and MDA contents (r=0,99).

4. DISCUSSION

The use of multiple biomarkers is highly recommended in environmental biomonitoring, to provide better estimates. Three different pollution biomarkers were applied to mussel hemolymph in this study – NRR assay, MDA contents and comet assay. Previous studies have been performed on the effect of WWTPs on live aquatic organisms. Pelletier *et al.* (2022) report the bioaccumulation of cyclic volatile methylsiloxanes and linear siloxanes in a food web in the St Lawrence River, Canada, downstream of the Montreal WWTP discharge. It is suggested that the effects may stem from the various chlorinated agents (chlorine gas, chlorine dioxide and sodium hypochlorite) used. These chemicals are highly effective in killing pathogenic microorganisms, but may also oxidize organic and inorganic matter, thus producing a variety of highly toxic, disinfection byproducts (DBPs) (Richardson *et al.* 2007). The characteristics of natural waters, such as the concentrations of natural organic matter (NOM), bromide or iodide, as well as other factors (pH, temperature), together with the type and quantity of disinfectant can all contribute to the formation of the final DBP mixtures (Singer 2008; Krasner 2009; Canistro *et al.* 2012). These mixtures may have adverse effects on marine organisms (Irving & Solbe 1980; Crathorne *et al.* 1992; Hutchinson *et al.* 1998), mainly because most halogenated DBPs are metabolized via the oxidative pathway cytochrome P450 (IPCS 2004). The metabolites generated can be connected with cellular components containing nucleophilic groups, including proteins, phospholipids and glutathione, causing cytotoxicity (Abbassi *et al.* 2010). Almost 50% of the daily WWTP input ends up in the sludge, the other 50% being released with the final effluent (Karvelas *et al.* 2003), suggesting that large WTTPs may be significant heavy metal sources for aquatic recipients. Abiotic factors (temperature, salinity, pH, oxygenation, water type and substrate) may also influence biomarker values (Vidal *et al.* 2002; Pfeifer *et al.* 2005).

NRR assay has been widely used as a water pollution biomarker to estimate the effects of pollutants. Aguirre-Martinez & Martín-Díaz (2020) measured lower NRR times in hemocytes of *R. philippinarum* exposed for 14 days at two sites close to WWTPs compared to those collected from a reference site. In this study, reduced NRR values were found in mussels treated with both WWTPE dilutions compared to control mussels. This is consistent with the results reported for clams (*R. philippinarum*), where NRR times decreased following exposure to undiluted, treated effluents (Maranho *et al.* 2015; Díaz-Garduño *et al.* 2018). Previous work in this laboratory supported the view that lysosomal membrane destabilization is caused by the oxidative action of both decontaminating agents and their by-products, resulting in the production of reactive oxygen species (ROSs) in the endolysosomal system. It is also possible that heavy metals, which remain in the treated wastewater at very low concentrations, have some effect on the biomarkers studied. The low NRR times found in this study could probably be attributed to increases in membrane permeability and loss of cytoplasm acid hydrolases, caused by heavy metals.

The lipid peroxidation results from this study are consistent with previous studies measurements of elevated MDA levels in clams (*R. decussatus*) under the influence of an urban WWTP, especially at the closest site (Silva *et al.* 2020). However, El Mourabit *et al.* (2022) reported no significant differences in MDA activity between a site close to a WWTP and two others characterized as clean. Aguirre-Martinez & Martín-Díaz (2020) found decreased LPO levels in *R. philippinarum* from the Bay of Cádiz, and raised levels in *C. fluminea* from the Guadalete River, indicating a complex WWTP effect. Gagné *et al.* (2007) measured increased LPO levels in *E. complanata* gill tissue exposed to different municipal effluent concentrations.

The comet assay results in this study showed elevated levels of DNA damage in mussels exposed to WWTPE compared to the controls. Previous studies showed that DNA damage increased in the digestive glands of *E. complanata* exposed to different concentrations of a primary-treated municipal effluent for seven weeks (Gagné *et al.* 2007). The results from this study are consistent with that, as well as with the study by Aguirre-Martinez & Martín-Díaz (2020) that indicated a higher effluent genotoxic effect through DNA damage in digestive gland tissues of *C. fluminea* exposed for 21 days, upstream and downstream of WWTPE discharges in the Guadalete River. The formation of DNA fragments could be one of the results, induced either indirectly via interaction with oxygen radicals or directly via inhibition of repair enzymes. This probably explains the elevated levels of DNA damage in mussels exposed to WWTPE compared to the controls, as shown by the comet assay.

Furthermore, elevated levels of DNA damage in mussels exposed to WWTPE may come from some interaction between the metals and proteins, leading to the formation of ROSs, which, in turn, react with DNA yielding single-strand fragments, as suggested by Kasprzak *et al.* (1992) and Misra *et al.* (1993).

5. CONCLUDING REMARKS

The results of this study support the roles of lysosomal membrane stability, MDA levels and DNA damage as pollution biomarkers in evaluating biological cleaning products using *M. galloprovincialis*. The exposure of freshwater mussels to chlorinated effluents reveals that treated municipal wastewaters have the potential to damage living organisms and possibly affect the status of the surrounding environment.

DECLARATIONS

The authors declare that they did not receive any funding for the present study. The authors declare that they have no known competing interests that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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